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APPLICATION NUMBER: 10/684,440

FILING DATE: October 15, 2003

RELATED PCT APPLICATION NUMBER: PCT/US04/14540

Certified by



Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. ISPH-0595P1.USA
First Inventor Crooke, et al.
Title Modulation of Apolipoprotein-
Express Mail Label No.

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Mail Stop Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

1. ☒ Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. ☐ Applicant claims small entity status.
See 37 CFR 1.27.
3. ☒ Specification [Total Pages 101]
(preferred arrangement set forth below)
 - Descriptive title of the invention
 - Cross Reference to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to sequence listing, a table, or a computer program listing appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
4. ☐ Drawing(s) (35 U.S.C. 113) [Total Sheets _____]
5. Oath or Declaration [Total Sheets _____]
 - a. ☐ Newly executed (original or copy)
 - b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 18 completed)
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s)
name in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☒ Application Data Sheet. See 37 CFR 1.76

7. ☐ CD-ROM or CD-R in duplicate, large table or Computer Program (Appendix)
8. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
 - a. ☒ Computer Readable Form (CRF)
 - b. Specification Sequence Listing on:
 - i. ☐ CD-ROM or CD-R (2 copies); or
 - ii. ☒ Paper
 - c. ☒ Statements verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

9. ☐ Assignment Papers (cover sheet & document(s))
10. ☐ 37 CFR 3.73(b) Statement of Attorney (when there is an assignee) ☐ Power of Attorney
11. ☐ English Translation Document (if applicable)
12. ☒ Information Disclosure Statement (IDS)/PTO-1449 ☒ Copies of IDS Citations
13. ☐ Preliminary Amendment
14. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
16. ☐ Nonpublication Request under 35 U.S.C. 122 (b)(2)(B)(i). Applicant must attach form PTO/SB/35 or its equivalent.
17. ☐ Other:

18. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application No.: 09/923,515.....

Prior application information: Examiner _____ Art Unit: _____
For CONTINUATION OF DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

19. CORRESPONDENCE ADDRESS

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Signature	Mary E. Bak	Date	Oct 15, 2003		

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT (\$) 1126.00

Complete If Known

Application Number	
Filing Date	Herewith
First Named Inventor	Crooke, et al.
Examiner Name	
Art Unit	
Attorney Docket No.	ISPH-0595US.P1

METHOD OF PAYMENT (check all that apply)

☒ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None☒ Deposit Account:
 Deposit Account Number
08-3040
 Deposit Account Name

The Director is authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☒ Credit any overpayments☒ Charge any additional fee(s) or any underpayment of fee(s)☒ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
1001 770	2001 385	Utility filing fee	770.00
1002 340	2002 170	Design filing fee	
1003 530	2003 265	Plant filing fee	
1004 770	2004 385	Reissue filing fee	
1005 160	2005 80	Provisional filing fee	
SUBTOTAL (1)			(\$) 770.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below	Fee Paid
Total Claims	35	-20** =	15	x 18	= 270
Independent Claims	4	-3** =	1	x 86	= 86
Multiple Dependent					

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description
1202 18	2202 9	Claims in excess of 20
1201 86	2201 43	Independent claims in excess of 3
1203 290	2203 145	Multiple dependent claim, if not paid
1204 86	2204 43	** Reissue independent claims over original patent
1205 18	2205 9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$) 356.00

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Small Entity

Fee Code (\$)	Fee Code (\$)	Fee Description	Fee Paid
1051 130	2051 65	Surcharge - late filing fee or oath	
1052 50	2052 25	Surcharge - late provisional filing fee or cover sheet	
1053 130	1053 130	Non-English specification	
1812 2,520	1812 2,520	For filing a request for ex parte reexamination	
1804 920*	1804 920*	Requesting publication of SIR prior to Examiner action	
1805 1,840*	1805 1,840*	Requesting publication of SIR after Examiner action	
1251 110	2251 55	Extension for reply within first month	
1252 420	2252 210	Extension for reply within second month	
1253 950	2253 475	Extension for reply within third month	
1254 1,480	2254 740	Extension for reply within fourth month	
1255 2,010	2255 1,005	Extension for reply within fifth month	
1401 330	2401 165	Notice of Appeal	
1402 330	2402 165	Filing a brief in support of an appeal	
1403 290	2403 145	Request for oral hearing	
1451 1,510	1451 1,510	Petition to institute a public use proceeding	
1452 110	2452 55	Petition to revive - unavoidable	
1453 1,330	2453 665	Petition to revive - unintentional	
1501 1,330	2501 665	Utility issue fee (or reissue)	
1502 480	2502 240	Design issue fee	
1503 640	2503 320	Plant issue fee	
1460 130	1460 130	Petitions to the Commissioner	
1807 50	1807 50	Processing fee under 37 CFR 1.17(q)	
1808 180	1808 180	Submission of Information Disclosure Stmt	
8021 40	8021 40	Recording each patent assignment per property (times number of properties)	
1809 770	2809 385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810 770	2810 385	For each additional invention to be examined (37 CFR 1.129(b))	
1801 770	2801 385	Request for Continued Examination (RCE)	
1802 900	1802 900	Request for expedited examination of a design application	

Other fee (specify)

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) (\$)

SUBMITTED BY

(Complete if applicable)

Name (Print/Type)	Mary E. Bak	Registration No. (Attorney/Agent)	31, 215	Telephone	215-540-9200
Signature	Mary E. Bak	Date	Oct 15, 2003		

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MODULATION OF APOLIPOPROTEIN(A) EXPRESSION**CROSS-REFERENCE TO RELATED APPLICATIONS**

5 This is a continuation-in-part application of pending United States patent application No. 09/923,515, filed August 7, 2001. This application also claims the benefit of the priority of United States provisional patent application No. 60/475,402, filed June 2, 2003.

10 BACKGROUND OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of Apolipoprotein(a).

15 Lipoproteins are globular, micelle-like particles that consist of a non-polar core of acylglycerols and cholesteryl esters, surrounded by an amphiphilic coating consisting of protein, phospholipid and cholesterol. Lipoproteins have been classified into five broad categories on the basis of their functional and physical properties: chylomicrons (which transport dietary lipids from intestine to tissues), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), (all of which transport triacylglycerols and cholesterol from the liver to 20 tissues), and high density lipoproteins (HDL) (which transport endogenous cholesterol from tissues to the liver).

Lipoprotein particles undergo continuous metabolic processing and have variable properties and compositions. 30 Lipoprotein densities increase without decreasing particle

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diameter because the density of their outer coatings is less than that of the inner core. The protein components of lipoproteins are known as apolipoproteins. At least nine apolipoproteins are distributed in significant amounts among the various human lipoproteins.

Lipoprotein(a) (also known as Lp(a)) is a cholesterol rich particle of the pro-atherogenic LDL class. Since Lp(a) is found only in Old World primates and European hedgehogs, it has been suggested that it does not play an essential role in lipid and lipoprotein metabolism. Most studies have shown that high concentrations of Lp(a) are strongly associated with increased risk of cardiovascular disease (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61). These observations have stimulated numerous studies in humans and other primates to investigate the factors that control Lp(a) concentrations and physiological properties (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61).

Lp(a) contains two disulfide-linked distinct proteins, apolipoprotein(a) (or ApoA) and apolipoprotein B (or ApoB) (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61). Apolipoprotein(a) is a unique apolipoprotein encoded by the LPA gene which has been shown to exclusively control the physiological concentrations of Lp(a) (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61). It varies in size due to interallelic differences in the number of tandemly repeated Kringle 4-encoding 5.5 kb sequences in the LPA gene (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61).

Cloning of human apolipoprotein(a) in 1987 revealed homology to human plasminogen (McLean et al., *Nature*, 1987, 330, 132-137). The gene locus LPA encoding

apolipoprotein(a) was localized to chromosome 6q26-27, in close proximity to the homologous gene for plasminogen (Frank et al., *Hum. Genet.*, 1988, 79, 352-356).

Transgenic mice expressing human Apolipoprotein(a) were found to be more susceptible than control mice to the development of lipid-staining lesions in the aorta. Consequently, apolipoprotein(a) is co-localized with lipid deposition in the artery walls (Lawn et al., *Nature*, 1992, 360, 670-672). As an extension of these studies, it was established that the major in vivo action of apolipoprotein(a) is inhibition of conversion of plasminogen to plasmin which causes decreased activation of latent transforming growth factor-beta. Since transforming growth factor-beta is a negative regulator of smooth muscle cell migration and proliferation, inhibition of plasminogen activation indicates a possible mechanism for apolipoprotein(a) induction of atherosclerotic lesions (Grainger et al., *Nature*, 1994, 370, 460-462).

Elevated plasma levels of Lp(a), caused by increased expression of apolipoprotein(a), are associated with increased risk for atherosclerosis and its manifestations, which include hypercholesterolemia (Seed et al., *N. Engl. J. Med.*, 1990, 322, 1494-1499), myocardial infarction (Sandkamp et al., *Clin. Chem.*, 1990, 36, 20-23), and thrombosis (Nowak-Gottl et al., *Pediatrics*, 1997, 99, E11).

Moreover, the plasma concentration of Lp(a) is strongly influenced by heritable factors and is refractory to most drug and dietary manipulation (Katan and Beynen, *Am. J. Epidemiol.*, 1987, 125, 387-399; Vessby et al., *Atherosclerosis*, 1982, 44, 61-71.). Pharmacologic therapy of elevated Lp(a) levels has been only modestly successful

and apheresis remains the most effective therapeutic modality (Hajjar and Nachman, *Annu. Rev. Med.*, 1996, 47, 423-442).

5 Morishita et al. have reported the use of three ribozyme oligonucleotides against apolipoprotein(a) for inhibition of apolipoprotein(a) expression in HepG2 cells (Morishita et al., *Circulation*, 1998, 98, 1898-1904).

10 US patent 5,721,138 refers to nucleotide sequences encoding the human apolipoprotein(a) gene 5'-regulatory region and isolated nucleotide sequences comprising at least thirty consecutive complementary nucleotides from human apolipoprotein(a) from nucleotide position -208 to -1448 (Lawn, 1998).

15 To date, investigative and therapeutic strategies aimed at inhibiting apolipoprotein(a) function have involved the previously cited use of Lp(a) apheresis and ribozyme oligonucleotides. Currently no existing drugs are available to specifically lower lipoprotein(a) levels in humans, and limited models exist in which to perform
20 drug discovery. Consequently, there remains a long-felt need for additional agents and methods capable of effectively modulating, e.g., inhibiting, apolipoprotein(a) function, and particularly a need for agents capable of safe and efficacious administration to
25 lower alipoprotein(a) levels in patients at risk for the development of coronary artery disease.

SUMMARY OF THE INVENTION

30 The present invention provides compositions and methods for modulating the expression of apolipoprotein(a). Such novel compositions and methods enable research into the pathways of plasminogen and

apolipoprotein(a), as well as other lipid metabolic processes. Such novel compositions and methods are useful in assessing the toxicity of chemical and pharmaceutical compounds on apolipoprotein(a) function, plasminogen or other lipid metabolic processes. Such novel compositions and methods are useful for drug discovery for the treatment of cardiovascular conditions, including myocardial infarction and atherosclerosis, among others.

5 In particular, this invention relates to compounds, particularly oligonucleotide compounds, which, in preferred embodiments, hybridize with nucleic acid molecules or sequences encoding apolipoprotein(a). Such compounds are shown herein to modulate the expression of apolipoprotein(a). Additionally disclosed are embodiments of oligonucleotide compounds that hybridize with nucleic acid molecules encoding apolipoprotein(a) in preference to nucleic acid molecules or sequences encoding plasminogen.

10 The present invention is directed to compounds, especially nucleic acid and nucleic acid-like oligomers, which are targeted to a nucleic acid encoding apolipoprotein(a), and which modulate the expression of apolipoprotein(a). Pharmaceutical and other compositions comprising the compounds of the invention are also provided.

20 Further provided are methods of screening for modulators of apolipoprotein(a) and methods of modulating the expression of apolipoprotein(a) in cells, tissues or animals comprising contacting said cells, tissues or animals with one or more of the compounds or compositions of the invention. Methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of

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apolipoprotein(a) are also set forth herein. Such methods
comprise administering a therapeutically or
prophylactically effective amount of one or more of the
compounds or compositions of the invention to the person
5 in need of treatment.

DETAILED DESCRIPTION OF THE INVENTION

A. Overview of the Invention

The present invention employs compounds,
10 preferably oligonucleotides and similar species, for use
in modulating the function or effect of nucleic acid
molecules encoding apolipoprotein(a). This is
accomplished by providing oligonucleotides which
specifically hybridize with one or more nucleic acid
15 molecules encoding apolipoprotein(a). As used herein, the
terms "target nucleic acid" and "nucleic acid molecule
encoding apolipoprotein(a)" have been used for convenience
to encompass DNA encoding apolipoprotein(a), RNA
(including pre-mRNA and mRNA or portions thereof)
20 transcribed from such DNA, and also cDNA derived from such
RNA. The hybridization of a compound of this invention
with its target nucleic acid is generally referred to as
"antisense". Antisense technology is emerging as an
effective means of reducing the expression of specific
25 gene products and is uniquely useful in a number of
therapeutic, diagnostic and research applications
involving modulation of Apolipoprotein(a) expression.

Consequently, the preferred mechanism believed to be
included in the practice of some preferred embodiments of
30 the invention is referred to herein as "antisense
inhibition." Such antisense inhibition is typically based
upon hydrogen bonding-based hybridization of

oligonucleotide strands or segments such that at least one strand or segment is cleaved, degraded, or otherwise rendered inoperable. In this regard, it is presently preferred to target specific nucleic acid molecules and their functions for such antisense inhibition.

The functions of DNA to be interfered with can include replication and transcription. Replication and transcription, for example, can be from an endogenous cellular template, a vector, a plasmid construct or otherwise. The functions of RNA to be interfered with can include functions such as translocation of the RNA to a site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more RNA species, and catalytic activity or complex formation involving the RNA which may be engaged in or facilitated by the RNA. One preferred result of such interference with target nucleic acid function is modulation of the expression of apolipoprotein(a). In the context of the present invention, "modulation" and "modulation of expression" mean either an increase (stimulation) or a decrease (inhibition) in the amount or levels of a nucleic acid molecule encoding the gene, e.g., DNA or RNA. Inhibition is often the preferred form of modulation of expression and mRNA is often a preferred target nucleic acid.

In the context of this invention, "hybridization" means the pairing of complementary strands of oligomeric compounds. In the present invention, the preferred mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide

bases (nucleobases) of the strands of oligomeric compounds. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds. Hybridization can occur under varying
5 circumstances.

An antisense compound is specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity, and there is a
10 sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target nucleic acid sequences under conditions in which specific binding is desired. Such conditions include, i.e., physiological conditions in the case of *in vivo* assays or therapeutic
15 treatment, and conditions in which assays are performed in the case of *in vitro* assays.

In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which a compound of the invention will
20 hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. In the context of this invention, "stringent conditions" under which oligomeric compounds
25 hybridize to a target sequence are determined by the nature and composition of the oligomeric compounds and the assays in which they are being investigated.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleobases of an
30 oligomeric compound. For example, if a nucleobase at a certain position of an oligonucleotide (an oligomeric compound), is capable of hydrogen bonding with a

nucleobase at a certain position of a target nucleic acid, said target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position. The oligonucleotide and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleobases that can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

The sequence of an antisense compound can be, but need not necessarily be, 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event. In one embodiment of this invention, the antisense compounds of the present invention comprise at least 70%, or at least 75%, or at least 80%, or at least 85% sequence complementarity to a target region within the target nucleic acid. In other embodiments, the antisense compounds of the present invention comprise at least 90% sequence complementarity and even comprise at least 95% or at least 99% sequence complementarity to the target region within the target nucleic acid sequence to which they are targeted. For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are

complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an antisense compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., *J. Mol. Biol.*, 1990, 215, 403-410; Zhang and Madden, *Genome Res.*, 1997, 7, 649-656).

Percent homology, sequence identity or complementarity, can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison WI), using default settings, which uses the algorithm of Smith and Waterman (*Adv. Appl. Math.*, 1981, 2, 482-489). In some preferred embodiments, homology, sequence identity or complementarity, between the oligomeric and target is between about 50% to about 60%. In some embodiments, homology, sequence identity or complementarity, is between about 60% to about 70%. In other embodiments, homology, sequence identity or complementarity, is between about 70% and about 80%. In still other embodiments, homology, sequence identity or

complementarity, is between about 80% and about 90%. In yet other embodiments, homology, sequence identity or complementarity, is about 90%, about 92%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%.

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B. Compounds of the Invention

According to the present invention, "compounds" include antisense oligomeric compounds, antisense oligonucleotides, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other oligomeric compounds that hybridize to at least a portion of the target nucleic acid. As such, these compounds may be introduced in the form of single-stranded, double-stranded, partially single-stranded, or circular oligomeric compounds. Specifically excluded from the definition of "compounds" herein are ribozymes that contain internal or external "bulges" that do not hybridize to the target sequence. Once introduced to a system, the compounds of the invention may elicit the action of one or more enzymes or structural proteins to effect modification of the target nucleic acid.

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One non-limiting example of such an enzyme is RNase H, a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense compounds which are "DNA-like" elicit RNase H. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. Similar roles have been postulated for other ribonucleases such as those in the RNase III and ribonuclease L family of enzymes.

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While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing.

The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, *Cell*, 1995, 81, 611-620). Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 15502-15507). The posttranscriptional antisense mechanism defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels (Fire et al., *Nature*, 1998, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs that are the potent inducers of RNAi (Tijsterman et al., *Science*, 2002, 295, 694-697).

The oligonucleotides of the present invention also include modified oligonucleotides in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, modified oligonucleotides may

be produced which contain thymidine, guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. These oligonucleotides are then tested using the methods described herein to
5 determine their ability to inhibit expression of apolipoprotein(a) mRNA.

In the context of this invention, the term "oligomeric compound" refers to a polymer or oligomer comprising a plurality of monomeric units. In the context
10 of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics, chimeras, analogs and homologs thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and
15 covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example,
20 enhanced cellular uptake, enhanced affinity for a target nucleic acid and increased stability in the presence of nucleases.

While oligonucleotides are a preferred form of the compounds of this invention, the present invention
25 comprehends other families of compounds as well, including but not limited to, oligonucleotide analogs and mimetics such as those described herein.

The compounds in accordance with this invention preferably comprise from about 8 to about 80 nucleobases
30 (i.e. from about 8 to about 80 linked nucleosides). One of ordinary skill in the art will appreciate that the invention embodies compounds of 8, 9, 10, 11, 12, 13, 14,

15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28,
29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42,
43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56,
57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70,
5 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleobases in
length.

In one preferred embodiment, the compounds of the
invention are 12 to 50 nucleobases in length. One having
ordinary skill in the art will appreciate that this
10 embodies compounds of 12, 13, 14, 15, 16, 17, 18, 19, 20,
21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34,
35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48,
49, or 50 nucleobases in length.

In another preferred embodiment, the compounds of the
15 invention are 15 to 30 nucleobases in length. One having
ordinary skill in the art will appreciate that this
embodies compounds of 15, 16, 17, 18, 19, 20, 21, 22, 23,
24, 25, 26, 27, 28, 29, or 30 nucleobases in length.

In one embodiment, compounds of this invention are
20 oligonucleotides from about 12 to about 50 nucleobases.
In another embodiment, compounds of this invention
comprise from about 15 to about 30 nucleobases.

Antisense compounds 8-80 nucleobases in length
comprising a stretch of at least eight (8) consecutive
25 nucleobases selected from within the illustrative
antisense compounds are considered to be suitable
antisense compounds as well.

Exemplary antisense compounds include oligonucleotide
sequences that comprise at least the 8 consecutive
30 nucleobases from the 5'-terminus of one of the
illustrative preferred antisense compounds (the remaining
nucleobases being a consecutive stretch of the same

oligonucleotide beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80 nucleobases). Similarly exemplary antisense compounds are represented by oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80 nucleobases).

Exemplary compounds of this invention may be found identified in the Examples and listed in Table 1. In addition to oligonucleotide compounds that bind to target sequences of apolipoprotein(a) in general, there are also exemplified oligonucleotide compounds of this invention that bind to target nucleotide sequences of apolipoprotein(a), but do not bind to, or do not bind preferentially to, sequences of plasminogen due to lack of homology between the two nucleic acid molecules or sufficient number of mismatches in the target sequences. These latter compounds are also useful in various therapeutic methods of this invention. Examples of antisense compounds to such 'mismatched' target sequences as described above include SEQ ID NO: 12 and SEQ ID NO: 23 of Table I below. See, also, the discussion of target regions below.

One having skill in the art armed with the exemplary antisense compounds illustrated herein will be able,

without undue experimentation, to identify further useful antisense compounds.

C. Targets of the Invention

5 "Targeting" an antisense compound to a particular nucleic acid molecule, in the context of this invention, can be a multistep process. The process usually begins with the identification of a target nucleic acid whose function is to be modulated. This target nucleic acid may
10 be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target nucleic acid encodes apolipoprotein(a).

15 The targeting process usually also includes determination of at least one target region, segment, or site within the target nucleic acid for the antisense interaction to occur such that the desired effect, e.g., modulation of expression, will result. Within the context
20 of the present invention, the term "region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic. Within regions of target nucleic acids are segments. "Segments" are defined as smaller or sub-portions of
25 regions within a target nucleic acid. "Sites," as used in the present invention, are defined as positions within a target nucleic acid.

 Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA
30 molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon".

A minority of genes has a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG; and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). Eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA transcribed from a gene encoding apolipoprotein(a), regardless of the sequence(s) of such codons. A translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively).

The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. Consequently, the "start codon region" (or "translation initiation codon region") and the "stop codon region" (or "translation termination

codon region") are all regions that may be targeted effectively with the antisense compounds of the present invention.

5 The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Within the context of the present invention, a preferred region is the intragenic region encompassing
10 the translation initiation or termination codon of the open reading frame (ORF) of a gene.

Another target region includes the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation
15 initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene). Still another target region includes the 3' untranslated region (3'UTR), known in the art to refer to the portion of an
20 mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene). The 5' cap site of an mRNA comprises an N7-methylated guanosine residue
25 joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap site. Another target region for this invention is the 5' cap
30 region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions,

known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. In one embodiment, targeting splice sites, i.e., intron-exon junctions or exon-intron junctions, is particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular splice product is implicated in disease. An aberrant fusion junction due to rearrangement or deletion is another embodiment of a target site. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". Introns can be effectively targeted using antisense compounds targeted to, for example, DNA or pre-mRNA.

Alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and exonic sequence.

Upon excision of one or more exon or intron regions, or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

Variants can be produced through the use of alternative signals to start or stop transcription. Pre-mRNAs and mRNAs can possess more than one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites. Within the context of the invention, the types of variants described herein are also embodiments of target nucleic acids.

The locations on the target nucleic acid to which the preferred antisense compounds hybridize are hereinbelow referred to as "preferred target segments." As used herein the term "preferred target segment" is defined as at least an 8-nucleobase portion of a target region to which an active antisense compound is targeted. While not wishing to be bound by theory, it is presently believed that these target segments represent portions of the target nucleic acid that are accessible for hybridization.

While the specific sequences of certain exemplary target segments are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional target segments are readily identifiable by one having ordinary skill in the art in view of this disclosure.

Target segments 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative preferred target segments are considered to be suitable for targeting as well.

Target segments can include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred target segments (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target segment and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly preferred target segments are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred target segments (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target segment and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art armed with the target segments illustrated herein will be able, without undue experimentation, to identify further preferred target segments.

Once one or more target regions, segments or sites have been identified, antisense compounds are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In various embodiments of this invention, the oligomeric compounds are targeted to regions of the target apollipoprotein(a) nucleobase sequence (e.g., such as

those disclosed in Example 13) comprising nucleobases 1-50, 51-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400, 401-450, 451-500, 501-550, 551-600, 601-650, 651-700, 701-750, 751-800, 801-850, 851-900, 901-950, 951-1000, 1001-1050, 1051-1100, 1101-1150, 1151-1200, 1201-1250, 1251-1300, 1301-1350, 1351-1400, 1401-1450, 1451-1500, 1501-1550, 1551-1600, 1601-1650, 1651-1700, 1701-1750, 1751-1800, 1801-1850, 1851-1900, 1901-1950, 1951-2000, 2001-2050, 2051-2100, 2101-2150, 2151-2200, 2201-2250, 2251-2300, 2301-2350, 2351-2400, 2401-2450, 2451-2500, 2501-2550, 2551-2600, 2601-2650, 2651-2700, 2701-2750, 2751-2800, 2801-2850, 2851-2900, 2901-2950, 2951-3000, 3001-3050, 3051-3100, 3101-3150, 3151-3200, 3201-3250, 3251-3300, 3301-3350, 3351-3400, 3401-3450, 3451-3500, 3501-3550, 3551-3600, 3601-3650, 3751-3700, 3701-3750, 3751-3800, 3801-3850, 3851-3900, 3901-3950, 3951-4000, 4001-4050, 4051-4100, 4101-4150, 4151-4200, 4201-4250, 4251-4300, 4301-4350, 4351-4400, 4401-4450, 4451-4500, 4501-4550, 4551-4600, 4601-4650, 4751-4700, 4701-4750, 4751-4800, 4801-4850, 4851-4900, 4901-4950, or 4951-5000, 5001-5050, 5051-5100, 5101-5150, 5151-5200, 5201-5250, 5251-5300, 5301-5350, 5351-5400, 5401-5450, 5451-5500, 5501-5550, 5551-5600, 5601-5650, 5651-5700, 5701-5750, 5751-5800, 5801-5850, 5851-5900, 5901-5950, 5951-6000, 6001-6050, 6051-6100, 6101-6150, 6151-6200, 6201-6250, 6251-6300, 6301-6350, 6351-6400, 6401-6450, 6451-6500, 6501-6550, 6551-6600, 6601-6650, 6651-6700, 6701-6750, 6751-6800, 6801-6850, 6851-6900, 6901-6950, 6951-7000, 7001-7050, 7051-7100, 7101-7150, 7151-7200, 7201-7250, 7251-7300, 7301-7350, 7351-7400, 7401-7450, 7451-7500, 7501-7550, 7551-7600, 7601-7650, 7651-7700, 7701-7750, 7751-7800, 7801-7850, 7851-7900, 7901-7950, 7951-

8000, 8001-8050, 8051-8100, 8101-8150, 8151-8200, 8201-8250, 8251-8300, 8301-8350, 8351-8400, 8401-8450, 8451-8500, 8501-8550, 8551-8600, 8601-8650, 8651-8700, 8701-8750, 8751-8800, 8801-8850, 8851-8900, 8901-8950, 8951-9000, 9001-9050, 9051-9100, 9101-9150, 9151-9200, 9201-9250, 9251-9300, 9301-9350, 9351-9400, 9401-9450, 9451-9500, 9501-9550, 9551-9600, 9601-9650, 9651-9700, 9701-9750, 9751-9800, 9801-9850, 9851-9900, 9901-9950, 9951-10000, 10001-10050, 10051-10100, 10101-10150, 10151-10200, 10201-10250, 10251-10300, 10301-10350, 10351-10400, 10401-10450, 10451-10500, 10501-10550, 10551-10600, 10601-10650, 10651-10700, 10701-10750, 10751-10800, 10801-10850, 10851-10900, 10901-10950, 10951-11000, 11001-11050, 11051-11100, 11101-11150, 11151-11200, 11201-11250, 11251-11300, 11301-11350, 11351-11400, 11401-11450, 11451-11500, 11501-11550, 11551-11600, 11601-11650, 11651-11700, 11701-11750, 11751-11800, 11801-11850, 11851-11900, 11901-11950, 11951-12000, 12001-12050, 12051-12100, 12101-12150, 12151-12200, 12201-12250, 12251-12300, 12301-12350, 12351-12400, 12401-12450, 12451-12500, 12501-12550, 12551-12600, 12601-12650, 12651-12700, 12701-12750, 12751-12800, 12801-12850, 12851-12900, 12901-12950, 12951-13000, 13001-13050, 13051-13100, 13101-13150, 13151-13200, 13201-13250, 13251-13300, 13301-13350, 13351-13400, 13401-13450, 13451-13500, 13501-13550, 13551-13600, 13601-13650, 13651-13700, 13701-13750, 13751-13800, 13801-13850, 13851-13900, 13901-13938, of apolipoprotein(a) or any combination thereof.

In one embodiment, the oligonucleotide compounds of this invention are 100% complementary to these sequences or to small sequences found within each of the above listed sequences. In another embodiment the oligonucleotide compounds have from at least 3 or 5

mismatches per 20 consecutive nucleobases in individual nucleobase positions to these target regions. Still other compounds of the invention are targeted to overlapping regions of the above-identified portions of the apolipoprotein(a) sequence.

In still another embodiment, target regions include those portions of the apolipoprotein(a) sequence that do not overlap with plasminogen sequences. For example, among such apolipoprotein(a) target sequences are included those found within the following nucleobase sequences: 10624-10702, 10963-11036, 11325-11354, 11615-11716, 11985-12038, 12319-12379, 13487-13491, and 13833-13871. As a further example, target sequences of apolipoprotein(a) that have at least 6 mismatches with the sequence of plasminogen over at least 20 consecutive nucleotides are desirable targets for antisense compounds that bind preferentially to apolipoprotein(a) rather than to plasminogen. Such target sequences can readily be identified by a BLAST comparison of the two GenBank sequences of plasminogen (e.g., GenBank Accession No. NM_000301) and apolipoprotein(a) (e.g., GenBank Accession No. NM_005577.1).

D. Screening and Target Validation

In a further embodiment, the "preferred target segments" identified herein may be employed in a screen for additional compounds that modulate the expression of apolipoprotein(a). "Modulators" are those compounds that decrease or increase the expression of a nucleic acid molecule encoding apolipoprotein(a) and which comprise at least an 8-nucleobase portion that is complementary to a preferred target segment. The screening method comprises

the steps of contacting a preferred target segment of a nucleic acid molecule encoding apolipoprotein(a) with one or more candidate modulators, and selecting for one or more candidate modulators which decrease or increase the expression of a nucleic acid molecule encoding apolipoprotein(a). Once it is shown that the candidate modulator or modulators are capable of modulating (e.g. either decreasing or increasing) the expression of a nucleic acid molecule encoding apolipoprotein(a), the modulator may then be employed in further investigative studies of the function of apolipoprotein(a), or for use as a research, diagnostic, or therapeutic agent in accordance with the present invention.

The preferred target segments of the present invention may be also be combined with their respective complementary antisense compounds of the present invention to form stabilized double-stranded (duplexed) oligonucleotides.

Such double stranded oligonucleotide moieties have been shown in the art to modulate target expression and regulate translation as well as RNA processing via an antisense mechanism. Moreover, the double-stranded moieties may be subject to chemical modifications (Fire et al., *Nature*, 1998, 391, 806-811; Timmons and Fire, *Nature* 1998, 395, 854; Timmons et al., *Gene*, 2001, 263, 103-112; Tabara et al., *Science*, 1998, 282, 430-431; Montgomery et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 15502-15507; Tuschl et al., *Genes Dev.*, 1999, 13, 3191-3197; Elbashir et al., *Nature*, 2001, 411, 494-498; Elbashir et al., *Genes Dev.* 2001, 15, 188-200). For example, such double-stranded moieties have been shown to inhibit the target by the classical hybridization of antisense strand of the duplex

to the target, thereby triggering enzymatic degradation of the target (Tijsterman et al., *Science*, 2002, 295, 694-697).

5 The compounds of the present invention can also be applied in the areas of drug discovery and target validation. The present invention comprehends the use of the compounds and preferred target segments identified herein in drug discovery efforts to elucidate relationships that exist between apolipoprotein(a) and a
10 disease state, phenotype, or condition. These methods include detecting or modulating apolipoprotein(a) comprising contacting a sample, tissue, cell, or organism with the compounds of the present invention, measuring the nucleic acid or protein level of apolipoprotein(a) and/or
15 a related phenotypic or chemical endpoint at some time after treatment, and optionally comparing the measured value to a non-treated sample or sample treated with a further compound of the invention. These methods can also be performed in parallel or in combination with other
20 experiments to determine the function of unknown genes for the process of target validation or to determine the validity of a particular gene product as a target for treatment or prevention of a particular disease, condition, or phenotype.

25

E. Kits, Research Reagents, Diagnostics, and Therapeutics

The compounds of the present invention can be utilized for diagnostics, therapeutics, and prophylaxis,
30 and as research reagents and components of kits. Furthermore, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are

often used by those of ordinary skill to elucidate the function of particular genes or to distinguish between functions of various members of a biological pathway.

For use in kits and diagnostics and in various biological systems, the compounds of the present invention, either alone or in combination with other compounds or therapeutics, are useful as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

As used herein the term "biological system" or "system" is defined as any organism, cell, cell culture or tissue that expresses, or is made competent to express products of the LPA gene. These include, but are not limited to, humans, transgenic animals, cells, cell cultures, tissues, xenografts, transplants and combinations thereof.

As one nonlimiting example, expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds that affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, 2000 480, 17-24; Celis, et al., *FEBS Lett.*, 2000 480, 2-16), SAGE (serial analysis of gene

expression) (Madden, et al., *Drug Discov. Today*, 2000, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, 1999, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Jungblut, et al., *Electrophoresis*, 1999, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Larsson, et al., *J. Biotechnol.*, 2000, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, 2000, 286, 91-98; Larson, et al., *Cytometry*, 2000, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, 2000, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, 1998, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, 1999, 35, 1895-904) and mass spectrometry methods (To, *Comb. Chem. High Throughput Screen*, 2000, 3, 235-41).

The compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding apolipoprotein(a). For example, oligonucleotides that hybridize with such efficiency and under such conditions as disclosed herein as to be effective apolipoprotein(a) inhibitors are effective primers or probes under conditions favoring gene amplification or detection, respectively. These primers and probes are useful in methods requiring the specific detection of nucleic acid molecules encoding apolipoprotein(a) and in the amplification of said nucleic acid molecules for detection or for use in further studies

of apolipoprotein(a). Hybridization of the antisense oligonucleotides, particularly the primers and probes, of the invention with a nucleic acid encoding apolipoprotein(a) can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide, or any other suitable detection means. Kits using such detection means for detecting the level of apolipoprotein(a) in a sample may also be prepared.

The specificity and sensitivity of antisense are also harnessed by those of skill in the art for therapeutic uses. Antisense compounds have been employed as therapeutic moieties in the treatment of disease states in animals, including humans. Antisense oligonucleotide drugs have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that antisense compounds can be useful therapeutic modalities that can be configured to be useful in treatment regimes for the treatment of cells, tissues and animals, especially humans.

For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of apolipoprotein(a) is treated by administering antisense compounds in accordance with this invention. For example, in one non-limiting embodiment, the methods comprise the step of administering to the animal in need of treatment, a therapeutically effective amount of a apolipoprotein(a) inhibitor. The apolipoprotein(a) inhibitors of the present invention effectively inhibit the activity of the apolipoprotein(a) protein or inhibit the expression of the

apolipoprotein(a) protein. In one embodiment, the activity or expression of apolipoprotein(a) in an animal is inhibited by about 10%. Preferably, the activity or expression of apolipoprotein(a) in an animal is inhibited by about 30%. More preferably, the activity or expression of apolipoprotein(a) in an animal is inhibited by 50% or more. Thus, the oligomeric compounds modulate expression of apolipoprotein(a) mRNA by at least 10%, by at least 20%, by at least 25%, by at least 30%, by at least 40%, by at least 50%, by at least 60%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 98%, by at least 99%, or by 100%.

For example, the reduction of the expression of apolipoprotein(a) may be measured in serum, adipose tissue, liver or any other body fluid, tissue or organ of the animal. Preferably, the cells contained within said fluids, tissues or organs being analyzed contain a nucleic acid molecule encoding apolipoprotein(a) protein and/or the apolipoprotein(a) protein itself. For example, apolipoprotein(a) is produced in the liver, and can be found in normal and atherosclerotic vessel walls.

The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of a compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the compounds and methods of the invention may also be useful prophylactically.

F. Modifications

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes

of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Modified Internucleoside Linkages (Backbones)

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in

their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphoro-
5 dithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-
10 amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked
15 analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide
20 linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages
25 include, but are not limited to, U.S. Patent Nos.:
3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196;
5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717;
5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233;
5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306;
30 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599;
5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050,

certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patent Nos.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Modified sugar and internucleoside linkages-Mimetics

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage (i.e. the backbone), of the nucleotide units are replaced with novel groups.

5 The nucleobase units are maintained for hybridization with an appropriate target nucleic acid. One such compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-
10 backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States
15 patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent Nos.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991,
20 254, 1497-1500.

Preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$ [known as a
25 methylene (methylimino) or MMI backbone], $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$ and $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$ [wherein the native phosphodiester backbone is represented as $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$] of the above referenced U.S. Patent No. 5,489,677, and the amide backbones of the above referenced U.S.
30 Patent No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent No. 5,034,506.

Modified sugars

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylamino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂), 2'-allyl (2'-CH₂-CH=CH₂), 2'-O-allyl (2'-O-CH₂-CH=CH₂) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up)

5 position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide.

10 Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but
15 are not limited to, U.S. Patent Nos.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920,
20 certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

A further preferred modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-
25 hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH₂-)_n group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in
30 published International Patent Application Nos. WO 98/39352 and WO 99/14226.

Natural and Modified Nucleobases

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in

which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent No. 3,687,808, as well as U.S. Patent Nos.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by

reference, and United States Patent No. 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

5 *Conjugates*

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Representative conjugate groups are disclosed in International Patent Application No. PCT/US92/09196, filed October 23, 1992, and U.S. Patent No. 6,287,860, the entire disclosures of which are

incorporated herein by reference. Conjugate moieties include, but are not limited to, lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethacin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999), which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide⁵ conjugates include, but are not limited to, U.S. Patent Nos.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022;

5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873;
5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463;
5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810;
5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696;
5 5,599,923; 5,599,928 and 5,688,941, certain of which are
commonly owned with the instant application, and each of
which is herein incorporated by reference.

Chimeric compounds

10 It is not necessary for all positions in a given
compound to be uniformly modified, and in fact more than
one of the aforementioned modifications may be
incorporated in a single compound or even at a single
nucleoside within an oligonucleotide.

15 The present invention also includes antisense
compounds that are chimeric compounds. "Chimeric"
antisense compounds or "chimeras," in the context of this
invention, are antisense compounds, particularly
oligonucleotides, which contain two or more chemically
20 distinct regions, each made up of at least one monomer
unit, i.e., a nucleotide in the case of an oligonucleotide
compound. These oligonucleotides typically contain at
least one region wherein the oligonucleotide is modified
so as to confer upon the oligonucleotide increased
25 resistance to nuclease degradation, increased cellular
uptake, increased stability and/or increased binding
affinity for the target nucleic acid. An additional
region of the oligonucleotide may serve as a substrate for
enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids.

30 By way of example, RNase H is a cellular endonuclease
which cleaves the RNA strand of an RNA:DNA duplex.
Activation of RNase H, therefore, results in cleavage of

the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. The cleavage of RNA:RNA hybrids can, in like fashion, be accomplished through the actions of endoribonucleases, such as RNaseL which cleaves both cellular and viral RNA. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patent Nos.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

G. Formulations

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not

limited to, U.S. Patent Nos.: 5,108,921; 5,354,844;
5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932;
5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556;
5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633;
5 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854;
5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948;
5,580,575; and 5,595,756, each of which is herein
incorporated by reference.

The antisense compounds of the invention encompass
10 any pharmaceutically acceptable salts, esters, or salts of
such esters, or any other compound which, upon
administration to an animal, including a human, is capable
of providing (directly or indirectly) the biologically
active metabolite or residue thereof.

15 The term "pharmaceutically acceptable salts" refers
to physiologically and pharmaceutically acceptable salts
of the compounds of the invention: i.e., salts that retain
the desired biological activity of the parent compound and
do not impart undesired toxicological effects thereto.
20 For oligonucleotides, preferred examples of
pharmaceutically acceptable salts and their uses are
further described in U.S. Patent No. 6,287,860, which is
incorporated herein in its entirety.

The present invention also includes pharmaceutical
25 compositions and formulations that include the antisense
compounds of the invention. The pharmaceutical
compositions of the present invention may be administered
in a number of ways depending upon whether local or
systemic treatment is desired and upon the area to be
30 treated. Administration may be topical (including
ophthalmic and to mucous membranes including vaginal and
rectal delivery), pulmonary, e.g., by inhalation or

insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be

formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations of the present invention may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. Emulsions may contain additional components in addition to the dispersed phases, and the active drug that may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as an embodiment of the present invention. Emulsions and their uses are well known in the art and are further described in U.S. Patent No. 6,287,860, which is incorporated herein in its entirety.

Formulations of the present invention include liposomal formulations. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are

positively charged liposomes that are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids. When incorporated into liposomes, these specialized lipids result in liposomes with enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. Liposomes and their uses are further described in U.S. Patent No. 6,287,860, which is incorporated herein in its entirety.

The pharmaceutical formulations and compositions of the present invention may also include surfactants. The use of surfactants in drug products, formulations and in emulsions is well known in the art. Surfactants and their uses are further described in U.S. Patent No. 6,287,860, which is incorporated herein in its entirety.

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five

broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Penetration enhancers and their uses are further described in U.S. Patent No. 6,287,860, which is
5 incorporated herein in its entirety.

One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

Preferred formulations for topical administration
10 include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoyl-
15 phosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoyl-phosphatidyl ethanolamine DOTMA).

20 For topical or other administration, oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids.
25 Preferred fatty acids and esters, pharmaceutically acceptable salts thereof, and their uses are further described in U.S. Patent No. 6,287,860, which is incorporated herein in its entirety. Topical formulations are described in detail in United States patent
30 application 09/315,298 filed on May 20, 1999, which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitabets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts and fatty acids and their uses are further described in U.S. Patent No. 6,287,860, which is incorporated herein in its entirety. Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents and their uses are further described in U.S. Patent No. 6,287,860, which is incorporated herein in its entirety. Oral formulations for oligonucleotides and their preparation are described in detail in U. S. Published Patent Application No. 2003/0040497 (Feb. 27, 2003) and its parent applications; U. S. Published Patent Application No. 2003/0027780 (Feb. 6, 2003) and its parent applications; and U. S. Patent Application No. 10/071,822, filed February 8, 2002, each

of which is incorporated herein by reference in their entirety.

5 Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

10 Certain embodiments of the invention provide pharmaceutical compositions containing one or more oligomeric compounds and one or more other chemotherapeutic agents that function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to cancer chemotherapeutic drugs such as daunorubicin, 15 daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, 20 testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptapurine, 6-thioguanine, cytarabine, 5-azacytidine, 25 hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). When used with the 30 compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide),

sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. Combinations of antisense compounds and other non-antisense drugs are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. For example, the first target may be a apolipoprotein(a) target, and the second target may be a region from another nucleotide sequence. Alternatively, compositions of the invention may contain two or more antisense compounds targeted to different regions of the same apolipoprotein(a) nucleic acid target. Numerous examples of antisense compounds are illustrated herein and others may be selected from among suitable compounds known in the art. Two or more combined compounds may be used together or sequentially:

H. Dosing

The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is dependent

on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50} s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μ g to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μ g to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same. Each of the references, GenBank accession numbers, as well as each application from which the present application claims priority, and the like recited in the

present application is incorporated herein by reference in its entirety.

EXAMPLES

5 Example 1

Synthesis of Nucleoside Phosphoramidites

The following compounds, including amidites and their intermediates were prepared as described in US Patent 6,426,220 and published International Patent Application
10 No. WO 02/36743; 5'-O-Dimethoxytrityl-thymidine intermediate for 5-methyl dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-5-methylcytidine intermediate for 5-methyl-dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-N⁴-benzoyl-5-methylcytidine penultimate intermediate for 5-methyl dC amidite, [5'-O-(4,4'-Dimethoxytriphenylmethyl)-
15 2'-deoxy-N⁴-benzoyl-5-methylcytidin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (5-methyl dC amidite), 2'-Fluorodeoxyadenosine, 2'-Fluorodeoxyguanosine, 2'-Fluorouridine, 2'-Fluorodeoxycytidine, 2'-O-(2-Methoxyethyl) modified amidites, 2'-O-(2-methoxyethyl)-5-methyluridine intermediate, 5'-O-DMT-2'-O-(2-methoxyethyl)-5-methyluridine penultimate intermediate, [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-5-methyluridin-3'-O-yl]-2-cyanoethyl-N,N-
20 diisopropylphosphoramidite (MOE T amidite), 5'-O-Dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methylcytidine intermediate, 5'-O-dimethoxytrityl-2'-O-(2-methoxyethyl)-N⁴-benzoyl-5-methyl-cytidine penultimate intermediate, [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N⁴-benzoyl-5-methylcytidin-3'-O-yl]-2-cyanoethyl-N,N-
25 diisopropylphosphoramidite (MOE 5-Me-C amidite), [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N⁶-

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benzoyladenoin-3'-O-yl]-2-cyanoethyl-N,N-
diisopropylphosphoramidite (MOE A amidite), [5'-O-(4,4'-
Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N⁴-
isobutyrylguanoin-3'-O-yl]-2-cyanoethyl-N,N-
5 diisopropylphosphoramidite (MOE G amidite), 2'-O-
(Aminooxyethyl) nucleoside amidites and 2'-O-
(dimethylaminooxyethyl) nucleoside amidites, 2'-
(Dimethylaminooxyethoxy) nucleoside amidites, 5'-O-tert-
Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine, 5'-O-
10 tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-
methyluridine, 2'-O-([2-phthalimidoxy)ethyl]-5'-t-
butyldiphenylsilyl-5-methyluridine, 5'-O-tert-
butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-
methyluridine, 5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N
15 dimethylaminooxyethyl]-5-methyluridine, 2'-O-
(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-
(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-(2-
N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-
cyanoethyl)-N,N-diisopropylphosphoramidite], 2'-
20 (Aminooxyethoxy) nucleoside amidites, N2-isobutyryl-6-O-
diphenylcarbamoyle-2'-O-(2-ethylacetyl)-5'-O-(4,4'-
dimethoxytrityl)guanoin-3'-[(2-cyanoethyl)-N,N-
diisopropylphosphoramidite], 2'-dimethylaminoethoxyethoxy
(2'-DMAEOE) nucleoside amidites, 2'-O-[2(2-N,N-
25 dimethylaminoethoxy)ethyl]-5-methyl uridine, 5'-O-
dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-
ethyl]-5-methyl uridine and 5'-O-Dimethoxytrityl-2'-O-
[2(2-N,N-dimethylaminoethoxy)-ethyl]-5-methyl uridine-3'-
O-(cyanoethyl-N,N-diisopropyl)phosphoramidite.

Example 2**Oligonucleotide and oligonucleoside synthesis**

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors, including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

Oligonucleotides: Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized similar to phosphodiester oligonucleotides with the following exceptions: thiation was effected by utilizing a 10% w/v solution of 3,4-dihydro-2H-benzothiole-3-one 1,1-dioxide in acetonitrile for the oxidation of the phosphite linkages. The thiation reaction step time was increased to 180 sec and preceded by the normal capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (12-16 hr), the oligonucleotides were recovered by precipitating with >3 volumes of ethanol from a 1M NH₄OAc solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent No. 4,469,863, herein incorporated by reference.

5 3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patent Nos. 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent No. 5,256,775 or 5,366,878, herein incorporated by reference.

10 Alkylphosphonothioate oligonucleotides are prepared as described in published International patent application Nos. PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

15 3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent No. 5,476,925, herein incorporated by reference.

20 Phosphotriester oligonucleotides are prepared as described in U.S. Patent No. 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patent Nos. 5,130,302 and 5,177,198, both herein incorporated by reference.

25 Oligonucleosides: Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having,

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for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patent Nos. 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

5 Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patent Nos. 5,264,562 and 5,264,564, herein incorporated by reference.

 Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent No. 5,223,618, herein
10 incorporated by reference.

Example 3

RNA Synthesis

 In general, RNA synthesis chemistry is based on the
15 selective incorporation of various protecting groups at strategic intermediary reactions. Although one of ordinary skill in the art will understand the use of protecting groups in organic synthesis, a useful class of protecting groups includes silyl ethers. In particular
20 bulky silyl ethers are used to protect the 5'-hydroxyl in combination with an acid-labile orthoester protecting group on the 2'-hydroxyl. This set of protecting groups is then used with standard solid-phase synthesis technology. It is important to lastly remove the acid labile
25 orthoester protecting group after all other synthetic steps. Moreover, the early use of the silyl protecting groups during synthesis ensures facile removal when desired, without undesired deprotection of 2' hydroxyl.

 Following this procedure for the sequential
30 protection of the 5'-hydroxyl in combination with protection of the 2'-hydroxyl by protecting groups that

are differentially removed and are differentially chemically labile, RNA oligonucleotides were synthesized.

RNA oligonucleotides are synthesized in a stepwise fashion. Each nucleotide is added sequentially (3'- to 5'-direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are added, coupling the second base onto the 5'-end of the first nucleoside. The support is washed and any unreacted 5'-hydroxyl groups are capped with acetic anhydride to yield 5'-acetyl moieties. The linkage is then oxidized to the more stable and ultimately desired P(V) linkage. At the end of the nucleotide addition cycle, the 5'-silyl group is cleaved with fluoride. The cycle is repeated for each subsequent nucleotide.

Following synthesis, the methyl protecting groups on the phosphates are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF. The deprotection solution is washed from the solid support-bound oligonucleotide using water. The support is then treated with 40% methylamine in water for 10 minutes at 55°C. This releases the RNA oligonucleotides into solution, deprotects the exocyclic amines, and modifies the 2'- groups. The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

The 2'-orthoester groups are the last protecting groups to be removed. The ethylene glycol monoacetate orthoester protecting group developed by Dharmacon Research, Inc. (Lafayette, CO), is one example of a useful orthoester protecting group that has the following important properties. It is stable to the conditions of

nucleoside phosphoramidite synthesis and oligonucleotide synthesis. However, after oligonucleotide synthesis the oligonucleotide is treated with methylamine, which not only cleaves the oligonucleotide from the solid support but also removes the acetyl groups from the orthoesters. The resulting 2-ethyl-hydroxyl substituents on the orthoester are less electron withdrawing than the acetylated precursor. As a result, the modified orthoester becomes more labile to acid-catalyzed hydrolysis. Specifically, the rate of cleavage is approximately 10 times faster after the acetyl groups are removed. Therefore, this orthoester possesses sufficient stability in order to be compatible with oligonucleotide synthesis and yet, when subsequently modified, permits deprotection to be carried out under relatively mild aqueous conditions compatible with the final RNA oligonucleotide product.

Additionally, methods of RNA synthesis are well known in the art (Scaringe, S. A. Ph.D. Thesis, University of Colorado, 1996; Scaringe, S. A., et al., *J. Am. Chem. Soc.*, **1998**, 120, 11820-11821; Matteucci, M. D. and Caruthers, M. H. *J. Am. Chem. Soc.*, **1981**, 103, 3185-3191; Beaucage, S. L. and Caruthers, M. H. *Tetrahedron Lett.*, **1981**, 22, 1859-1862; Dahl, B. J., et al., *Acta Chem. Scand.*, **1990**, 44, 639-641; Reddy, M. P., et al., *Tetrahedron Lett.*, **1994**, 25, 4311-4314; Wincott, F. et al., *Nucleic Acids Res.*, **1995**, 23, 2677-2684; Griffin, B. E., et al., *Tetrahedron*, **1967**, 23, 2301-2313; Griffin, B. E., et al., *Tetrahedron*, **1967**, 23, 2315-2331).

RNA antisense compounds (RNA oligonucleotides) of the present invention can be synthesized by the methods herein or purchased from Dharmacon Research, Inc (Lafayette, CO).

Once synthesized, complementary RNA antisense compounds can then be annealed by methods known in the art to form double stranded (duplexed) antisense compounds. For example, duplexes can be formed by combining 30 μ l of each of the complementary strands of RNA oligonucleotides (50 μ M RNA oligonucleotide solution) and 15 μ l of 5X annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) followed by heating for 1 minute at 90°C, then 1 hour at 37°C. The resulting duplexed antisense compounds can be used in kits, assays, screens, or other methods to investigate the role of a target nucleic acid.

Example 4

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 394, as above.

Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by incorporating coupling steps with increased reaction times for the 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite. The fully protected oligonucleotide is cleaved from the support and deprotected in concentrated ammonia (NH₄OH) for 12-16 hr at 55°C. The deprotected oligo is then recovered by an appropriate method (precipitation, column chromatography, volume reduced *in vacuo* and analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

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[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

25

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for

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the 2'-O-methyl amidites, oxidation with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States Patent No. 5,623,065, herein incorporated by reference.

Example 5

Design and screening of duplexed antisense compounds targeting apolipoprotein(a)

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target apolipoprotein(a). The nucleobase sequence of the antisense strand of the duplex comprises at least an 8-nucleobase portion of an oligonucleotide in Table 1. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini.

For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

```
5          cgagaggcggacgggaccgTT      Antisense Strand
          |||||
          TTgctctccgcctgccctggc      Complement
```

10 RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 μ M. Once diluted, 30 μ L of each strand is combined with 15 μ L
15 of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 μ L. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The
20 tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 μ M. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

25 Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate apolipoprotein(a) expression.

When cells reached 80% confluency, they are treated with duplexed antisense compounds of the invention. For
30 cells grown in 96-well plates, wells are washed once with 200 μ L OPTI-MEM-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of OPTI-MEM-1 containing 12 μ g/mL LIPOFECTIN reagent (Gibco BRL) and the desired duplex antisense compound at a final concentration of 200 nM.

After 5 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after treatment, at which time RNA is isolated and target reduction measured by RT-PCR.

5

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass solid support and deblocking in concentrated ammonium hydroxide at 55°C for 12-16 hours, the oligonucleotides or oligonucleosides are recovered by precipitation out of 1 M NH₄OAc with >3 volumes of ethanol. Synthesized oligonucleotides were analyzed by electrospray mass spectroscopy (molecular weight determination) and by capillary gel electrophoresis and judged to be at least 70% full-length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in the synthesis were determined by the ratio of correct molecular weight relative to the -16 amu product (+/-32 +/-48). For some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

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Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a 96-well format. Phosphodiester internucleotide linkages were afforded by oxidation with

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aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per standard or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature ($55-60^\circ\text{C}$) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96-Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96-well format (Beckman P/ACETM MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACETM 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were

judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

5 Cell culture and oligonucleotide treatment

The effects of antisense compounds on target nucleic acid expression are tested in any of a variety of cell types, provided that the target nucleic acid is present at measurable levels. This can be routinely determined
10 using, for example, PCR or Northern blot analysis. The following cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine
15 in the art, for example Northern blot analysis, ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell
20 line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad,
25 CA), penicillin 100 units per mL, and streptomycin 100 µg/mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #353872) at a density of 7000
30 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates.

and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

5 The human lung carcinoma cell line A549 was obtained
from the American Type Culture Collection (ATCC)
(Manassas, VA). A549 cells were routinely cultured in
DMEM basal media (Invitrogen Corporation, Carlsbad, CA)
supplemented with 10% fetal calf serum (Invitrogen
10 Corporation, Carlsbad, CA), penicillin 100 units per mL,
and streptomycin 100 µg/mL (Invitrogen Corporation,
Carlsbad, CA). Cells were routinely passaged by
trypsinization and dilution when they reached 90%
confluence.

15

NHDF cells:

Human neonatal dermal fibroblasts (NHDFs) were
obtained from the Clonetics Corporation (Walkersville,
MD). NHDFs were routinely maintained in Fibroblast Growth
20 Medium (Clonetics Corporation, Walkersville, MD)
supplemented as recommended by the supplier. Cells were
maintained for up to 10 passages as recommended by the
supplier.

25 HEK cells:

Human embryonic keratinocytes (HEK) were obtained
from the Clonetics Corporation (Walkersville, MD). HEKs
were routinely maintained in Keratinocyte Growth Medium
(Clonetics Corporation, Walkersville, MD) formulated as
30 recommended by the supplier. Cells were routinely
maintained for up to 10 passages as recommended by the
supplier.

Treatment with antisense compounds:

When cells reached 65-75% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 100 μ L OPTI-MEM™-1 reduced-serum medium (Invitrogen Corporation, Carlsbad, CA) and then treated with 130 μ L of OPTI-MEM™-1 medium containing 3.75 μ g/mL LIPOFECTIN™ reagent (Invitrogen Corporation, Carlsbad, CA) and the desired concentration of oligonucleotide. Cells are treated and data are obtained in triplicate. After 4-7 hours of treatment at 37°C, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is selected from either ISIS 13920 (**TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1) which is targeted to human H-ras, or ISIS 18078, (**GTGCGCGCGAGCCCGAAATC**, SEQ ID NO: 2) which is targeted to human Jun-N-terminal kinase-2 (JNK2). Both controls are 2'-O-methoxyethyl gapmers (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCCAAGGA**, SEQ ID NO: 3, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-H-ras (for ISIS 13920), JNK2 (for ISIS 18078) or c-raf (for ISIS

15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of c-H-ras, JNK2 or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments. The concentrations of antisense oligonucleotides used herein are from 50 nM to 300 nM.

Example 10

Analysis of oligonucleotide inhibition of apolipoprotein(a) expression

Antisense modulation of apolipoprotein(a) expression can be assayed in a variety of ways known in the art. For example, apolipoprotein(a) mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. The preferred method of RNA analysis of the present invention is the use of total cellular RNA as described in other examples herein. Methods of RNA isolation are well known in the art. Northern blot analysis is also routine in the art. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7600, 7700, or 7900 Sequence Detection System, available from PE-Applied

Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of apolipoprotein(a) can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS). Antibodies directed to apolipoprotein(a) can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional monoclonal or polyclonal antibody generation methods well known in the art.

Example 11

Design of phenotypic assays and in vivo studies for the use of apolipoprotein(a) inhibitors

Phenotypic assays

Once apolipoprotein(a) inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition. Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of apolipoprotein(a) in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic

assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

10 In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with apolipoprotein(a) inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

20 Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status, which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest.

25 Analysis of the genotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the apolipoprotein(a) inhibitors. Hallmark genes, or those genes suspected to be associated

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with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

The cells subjected to the phenotypic assays described herein derive from in vitro cultures or from
5 tissues or fluids isolated from living organisms, both human and non-human. In certain embodiments, a tissue and its constituent cells comprise, but are not limited to, blood (e.g., hematopoietic cells, such as human hematopoietic progenitor cells, human hematopoietic stem
10 cells, CD34⁺ cells CD4⁺ cells), lymphocytes and other blood lineage cells, bone marrow, brain, stem cells, blood vessel, liver, lung, bone, breast, cartilage, cervix, colon, cornea, embryonic, endometrium, endothelial, epithelial, esophagus, fascia, fibroblast, follicular,
15 ganglion cells, glial cells, goblet cells, kidney, lymph node, muscle, neuron, ovaries, pancreas, peripheral blood, prostate, skin, skin, small intestine, spleen, stomach, testes and fetal tissue. In other embodiments, a fluid and its constituent cells comprise, but is not limited to,
20 blood, urine, synovial fluid, lymphatic fluid and cerebrospinal fluid. The phenotypic assays may also be performed on tissues treated with apolipoprotein(a) inhibitors *ex vivo*.

25 *In vivo studies*

The individual subjects of the *in vivo* studies described herein are warm-blooded vertebrate animals, including humans.

The clinical trial is subjected to rigorous controls
30 to ensure that individuals are not unnecessarily put at risk and that they are fully informed about their role in the study.

To account for the psychological effects of receiving treatments, volunteers are randomly given placebo or apolipoprotein(a) inhibitor. Furthermore, to prevent the doctors from being biased in treatments, they are not
5 informed as to whether the medication they are administering is a apolipoprotein(a) inhibitor or a placebo. Using this randomization approach, each volunteer has the same chance of being given either the new treatment or the placebo.

10 Volunteers receive either the apolipoprotein(a) inhibitor or placebo for eight week period with biological parameters associated with the indicated disease state or condition being measured at the beginning (baseline measurements before any treatment), end (after the final
15 treatment), and at regular intervals during the study period. Such measurements include the levels of nucleic acid molecules encoding apolipoprotein(a) or apolipoprotein(a) protein levels in body fluids, tissues or organs compared to pre-treatment levels. Other
20 measurements include, but are not limited to, indices of the disease state or condition being treated, body weight, blood pressure, serum titers of pharmacologic indicators of disease or toxicity as well as ADME (absorption, distribution, metabolism and excretion) measurements.

25 Information recorded for each patient includes age (years), gender, height (cm), family history of disease state or condition (yes/no), motivation rating (some/moderate/ great) and number and type of previous treatment regimens for the indicated disease or condition.

30 Volunteers taking part in this study are healthy adults (age 18 to 65 years) and roughly an equal number of males and females participate in the study. Volunteers

with certain characteristics are equally distributed for placebo and apolipoprotein(a) inhibitor treatment. In general, the volunteers treated with placebo have little or no response to treatment, whereas the volunteers
5 treated with the apolipoprotein(a) inhibitor show positive trends in their disease state or condition index at the conclusion of the study.

Example 12

10 RNA Isolation

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura *et al.*, (*Clin. Chem.*, 1996, 42, 1758-1764). Other methods for poly(A)+ mRNA isolation are routine in the art. Briefly,
15 for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-
ribonucleoside complex) was added to each well, the plate
20 was gently agitated and then incubated at room temperature for five minutes. 55 μ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed
3 times with 200 μ L of wash buffer (10 mM Tris-HCl pH 7.6,
25 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C, was added to
each well, the plate was incubated on a 90°C hot plate for
30 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

5 *Total RNA Isolation*

 Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium
10 was removed from the cells and each well was washed with 200 µL cold PBS. 150 µL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 150 µL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The
15 samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 1 minute. 500 µL of Buffer RW1 was added to each well of the RNEASY 96™ plate and incubated for 15
20 minutes and the vacuum was again applied for 1 minute. An additional 500 µL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum was applied for 2 minutes. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a
25 period of 90 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 3 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a
30 collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 140 µL of RNase free

water into each well, incubating 1 minute, and then applying the vacuum for 3 minutes.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13

Real-time Quantitative PCR Analysis of apolipoprotein(a) mRNA Levels

Quantitation of apolipoprotein(a) mRNA levels was accomplished by real-time quantitative PCR using the ABI PRISM™ 7600, 7700, or 7900 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., FAM or JOE, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies

Inc., Alameda, CA or Integrated DNA Technologies Inc.,
Coralville, IA) is attached to the 3' end of the probe.
When the probe and dyes are intact, reporter dye emission
is quenched by the proximity of the 3' quencher dye.

5 During amplification, annealing of the probe to the target
sequence creates a substrate that can be cleaved by the
5'-exonuclease activity of Taq polymerase. During the
extension phase of the PCR amplification cycle, cleavage
of the probe by Taq polymerase releases the reporter dye
10 from the remainder of the probe (and hence from the
quencher moiety) and a sequence-specific fluorescent
signal is generated. With each cycle, additional reporter
dye molecules are cleaved from their respective probes,
and the fluorescence intensity is monitored at regular
15 intervals by laser optics built into the ABI PRISM™
Sequence Detection System. In each assay, a series of
parallel reactions containing serial dilutions of mRNA
from untreated control samples generates a standard curve
that is used to quantitate the percent inhibition after
20 antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets
specific to the target gene being measured are evaluated
for their ability to be "multiplexed" with a GAPDH
amplification reaction. In multiplexing, both the target
25 gene and the internal standard gene GAPDH are amplified
concurrently in a single sample. In this analysis, mRNA
isolated from untreated cells is serially diluted. Each
dilution is amplified in the presence of primer-probe sets
specific for GAPDH only, target gene only ("single-
30 plexing"), or both (multiplexing). Following PCR
amplification, standard curves of GAPDH and target mRNA
signal as a function of dilution are generated from both

the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from Invitrogen Corporation, (Carlsbad, CA). RT-PCR reactions were carried out by adding 20 μ L PCR cocktail (2.5x PCR buffer minus $MgCl_2$, 6.6 mM $MgCl_2$, 375 μ M each of dATP, dCTP, dGTP and dTTP, 375 nM each of forward primer and reverse primer, 125 nM of probe, 4 Units RNase inhibitor, 1.25 Units PLATINUM® Taq, 5 Units MuLV reverse transcriptase, and 2.5x ROX dye) to 96-well plates containing 30 μ L total RNA solution (20-200 ng). The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the PLATINUM® Taq, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent (Molecular Probes, Inc. Eugene, OR). Methods of RNA quantification by RiboGreen™ reagent

are taught in Jones, L.J., et al, (*Analytical Biochemistry*, 1998, 265, 368-374).

In this assay, 170 μ L of RiboGreenTM working reagent (RiboGreenTM reagent diluted 1:350 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 30 μ L purified, cellular RNA. The plate is read in a CytoFluor 4000 apparatus (PE Applied Biosystems) with excitation at 485nm and emission at 530nm.

Probes and primers to human apolipoprotein(a) were designed to hybridize to a human apolipoprotein(a) sequence, using published sequence information (GenBank accession number NM_005577.1, incorporated herein as SEQ ID NO: 4). For human apolipoprotein(a) the PCR primers were:

forward primer: CAGCTCCTTATTGTTATACGAGGGA (SEQ ID NO: 5)
reverse primer: TCGTCTGAGCATTGCGT (SEQ ID NO: 6) and the PCR probe was: FAM-CCCGGTGTCAGGTGGGAGTACTGC-TAMRA (SEQ ID NO: 7) where FAM is the fluorescent dye and TAMRA is the quencher dye. For human GAPDH the PCR primers

were:
forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 8)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 9) and the PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA 3' (SEQ ID NO: 10) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

Example 14

Northern blot analysis of apolipoprotein(a) mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOLTM (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended

protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 apparatus (Stratagene, Inc, La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human apolipoprotein(a), a human apolipoprotein(a) specific probe was prepared by PCR using the forward primer CAGCTCCTTATTGTTATACGAGGGA (SEQ ID NO: 5) and the reverse primer TGCCTCTGAGCATTGCGT (SEQ ID NO: 6). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ apparatus and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

Antisense inhibition of human apolipoprotein(a) expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

5 In accordance with the present invention, a series of antisense compounds was designed to target different regions of the human apolipoprotein(a) RNA, using published sequences (GenBank accession number NM_005577.1, incorporated herein as SEQ ID NO: 4). The compounds are
10 shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the compound binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of
15 ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide.
20 All cytidine residues are 5-methylcytidines.

 Apolipoprotein(a) is found in humans, nonhuman primates and the European hedgehog, but not in common laboratory animals such as rats and mice. Transgenic mice which express human apolipoprotein(a) have been engineered
25 (Chiesa et al., J. Biol. Chem., 1992, 267, 24369-24374). The use of primary hepatocytes prepared from human apolipoprotein(a) transgenic mice circumvents the issue of variability when testing antisense oligonucleotide activity in primary human hepatocytes. Accordingly,
30 primary mouse hepatocytes prepared from the human apolipoprotein(a) transgenic mice were used to investigate the effects of antisense oligonucleotides on human

apolipoprotein(a) expression. The human apolipoprotein(a) transgenic mice were obtained from Dr. Robert Pitas and Dr. Matthias Schneider in the Gladstone Institute at the University of California, San Francisco. Primary

5 hepatocytes were isolated from these mice and were cultured in DMEM, high glucose (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum, (Invitrogen Corporation, Carlsbad, CA), 100 units per mL penicillin/100 µg/mL streptomycin (Invitrogen Corporation,

10 Carlsbad, CA). For treatment with oligonucleotide, cells were washed once with serum-free DMEM and subsequently transfected with a dose of 150 nM of antisense oligonucleotide using LIPOFECTIN reagent (Invitrogen Corporation, Carlsbad, CA) as described in other examples

15 herein. The compounds were analyzed for their effect on human apolipoprotein(a) mRNA levels by quantitative real-time PCR as described in other examples herein. Gene target quantities obtained by real time RT-PCR were normalized using mouse GAPDH. For mouse GAPDH the PCR

20 primers were:
forward primer: GGCAAATTCAACGGCACAGT(SEQ ID NO: 71)
reverse primer: GGGTCTCGCTCCTGGAAGAT(SEQ ID NO: 72) and
the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC-TAMRA 3' (SEQ ID NO: 73) where JOE is the fluorescent

25 reporter dye and TAMRA is the quencher dye.

Data are averages from three experiments in which primary transgenic mouse hepatocytes were treated with the antisense oligonucleotides of the present invention.

Table 1

Inhibition of human apolipoprotein(a) mRNA levels by
chimeric phosphorothioate oligonucleotides having 2'-MOE
wings and a deoxy gap

5

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
144367	Coding	4	174	ggcaggtccttctctgtgaca	53	11
144368	Coding	4	352	tctgcgtctgagcattgcgt	87	12
144369	Coding	4	522	aagcttggcaggttcttct	0	13
144370	Coding	4	1743	tcggaggcgcgacggcagtc	40	14
144371	Coding	4	2768	cggaggcgcgacggcagtc	0	15
144372	Coding	4	2910	ggcaggttcttctctgtgaca	65	16
144373	Coding	4	3371	ataacaataaggagctgcc	50	17
144374	Coding	4	4972	gaccaagcttggcaggttct	62	18
144375	Coding	4	5080	taacaataaggagctgccac	36	19
144376	Coding	4	5315	tgaccaagcttggcaggttc	25	20
144377	Coding	4	5825	ttctgcgtctgagcattgcg	38	21
144378	Coding	4	6447	aacaataaggagctgccaca	29	22
144379	Coding	4	7155	acctgacaccgggatccctc	79	23
144380	Coding	4	7185	ctgagcattgcgtcaggttg	16	24
144381	Coding	4	8463	agtagttcatgatcaagcca	71	25
144382	Coding	4	8915	gacggcagtccttctgcgt	34	26
144383	Coding	4	9066	ggcaggttcttccagtgaca	5	27
144384	Coding	4	10787	tgaccaagcttggcaagttc	31	28
144385	Coding	4	11238	tataacaccaaggactaatc	9	29
144386	Coding	4	11261	ccatctgacattgggatcca	66	30
144387	Coding	4	11461	tgtggtgtcatagaggacca	36	31
144388	Coding	4	11823	atgggatcctccgatgccaa	55	32
144389	Coding	4	11894	acaccaaggcgcaatctcag	58	33
144390	Coding	4	11957	ttctgtcaactggacatcgtg	59	34
144391	Coding	4	12255	cacacggatcggttgtgtaa	58	35
144392	Coding	4	12461	acatgtccttctctgtgacag	51	36
144393	Coding	4	12699	cagaaggaggccctaggctt	33	37
144394	Coding	4	13354	ctggcggtgaccatgtagtc	52	38
144395	3'UTR	4	13711	tctaagtaggttgatgcttc	68	39
144396	3'UTR	4	13731	tccttaccacagtttcagct	70	40
144397	3'UTR	4	13780	ggaacagtgtcttcgtttga	63	41
144398	3'UTR	4	13801	gtttggcatagctggtagct	44	42
144399	3'UTR	4	13841	accttaaaagcttatacaca	57	43
144400	3'UTR	4	13861	atacagaatttgcagtcag	21	44
144401	3'UTR	4	13881	gtcatagctatgacacctta	46	45

As shown in Table 1, SEQ ID NOs 11, 12, 14, 16, 17,
18, 19, 21, 23, 25, 30, 31, 32, 33, 34, 35, 36, 38, 39,
40, 41, 42, 43 and 45 demonstrated at least 35% inhibition
of human apolipoprotein(a) expression in this assay and

10

are therefore preferred. More preferred are SEQ ID NOs 23, 12 and 40. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target segments are shown in Table 2. These sequences are shown to contain thymine (T) but one of skill in the art will appreciate that thymine (T) is generally replaced by uracil (U) in RNA sequences. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 2 is the species in which each of the preferred target segments was found.

Table 2
Sequence and position of preferred target segments
identified in apolipoprotein(a).

SITE ID	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	REV COMP OF SEQ ID	ACTIVE IN	SEQ ID NO
57364	4	174	tgtcacaggaaggacctgcc	11	<i>H. sapiens</i>	46
57365	4	352	acgcaatgctcagacgcaga	12	<i>H. sapiens</i>	47
57367	4	1743	gactgccgtcgcgcctccga	14	<i>H. sapiens</i>	48
57369	4	2910	tgtcacaggaagaacctgcc	16	<i>H. sapiens</i>	49
57370	4	3371	tggcagctccttattgttat	17	<i>H. sapiens</i>	50
57371	4	4972	agaacctgccaaagcttggtc	18	<i>H. sapiens</i>	51
57372	4	5080	gtggcagctccttattgtta	19	<i>H. sapiens</i>	52
57374	4	5825	cgcaatgctcagacgcagaa	21	<i>H. sapiens</i>	53
57376	4	7155	gagggatcccgggtgtcaggt	23	<i>H. sapiens</i>	54
57378	4	8463	tggcttgatcatgaactact	25	<i>H. sapiens</i>	55
57383	4	11261	tggatcccaatgtcagatgg	30	<i>H. sapiens</i>	56
57384	4	11461	tggctcctctatgacaccaca	31	<i>H. sapiens</i>	57
57385	4	11823	ttggcatcggaggatcccat	32	<i>H. sapiens</i>	58
57386	4	11894	ctgagattcggccttggtgt	33	<i>H. sapiens</i>	59
57387	4	11957	cacgatgtccagtgcagaaa	34	<i>H. sapiens</i>	60
57388	4	12255	ttacacaaccgatccgtgtg	35	<i>H. sapiens</i>	61
57389	4	12461	ctgtcacaggaaggacatgt	36	<i>H. sapiens</i>	62
57391	4	13354	gactacatggtcaccgccag	38	<i>H. sapiens</i>	63
57392	4	13711	gaagcatcaacctacttaga	39	<i>H. sapiens</i>	64
57393	4	13731	agctgaaacgtgggtaagga	40	<i>H. sapiens</i>	65
57394	4	13780	tcaaacgaagacactgttcc	41	<i>H. sapiens</i>	66
57395	4	13801	agctaccagctatgccaaac	42	<i>H. sapiens</i>	67
57396	4	13841	tgtgtataagcttttaaggt	43	<i>H. sapiens</i>	68
57398	4	13881	taagggtgtcatagctatgac	45	<i>H. sapiens</i>	69

5

As these "preferred target segments" have been found by experimentation to be open to, and accessible for, hybridization with the antisense compounds of the present invention, one of skill in the art will recognize or be able to ascertain, using no more than routine experimentation, further embodiments of the invention that encompass other compounds that specifically hybridize to these preferred target segments and consequently inhibit the expression of apolipoprotein(a).

10

15

According to the present invention, antisense compounds include antisense oligomeric compounds, antisense oligonucleotides, external guide sequence (EGS)

oligonucleotides, alternate splicers, primers, probes, and other short oligomeric compounds that hybridize to at least a portion of the target nucleic acid.

5 **Example 16**

Western blot analysis of apolipoprotein(a) protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with
10 PBS, suspended in Laemmli buffer (100 µl/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to apolipoprotein(a) is used, with a radiolabeled or
15 fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ apparatus (Molecular Dynamics, Sunnyvale CA).

20 **Example 17**

Antisense inhibition of human apolipoprotein(a) in transgenic primary mouse hepatocytes: dose response

In accordance with the present invention, antisense oligonucleotides identified as having good activity based
25 on the results in Example 15 were further investigated in dose-response studies. Primary hepatocytes from human apolipoprotein(a) transgenic mice were treated with 10, 50, 150 or 300 nM of ISIS 144396 (SEQ ID NO: 40), ISIS 144368 (SEQ ID NO: 12), ISIS 144379 (SEQ ID NO: 23) or
30 ISIS 113529 (CTCTTACTGTGCTGTGGACA, SEQ ID NO: 70). ISIS 113529 was used as a scrambled control oligonucleotide and is a chimeric oligonucleotides ("gapmers") 20 nucleotides

in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

Following 24 hours of exposure to antisense oligonucleotides, target mRNA expression levels were evaluated by quantitative real-time PCR as described in other examples herein. The results are the average of 4 experiments for apolipoprotein(a) antisense oligonucleotides and the average of 12 experiments for the control oligonucleotide. The data are expressed as percent inhibition of apolipoprotein(a) expression relative to untreated controls and are shown in Table 3.

Table 3

Antisense inhibition of human apolipoprotein(a) in transgenic primary mouse hepatocytes: dose response

Oligonucleotide dose	% Inhibition of transgenic human lipoprotein(a)			
	ISIS #			
	144396	144368	144379	113529
10 nM	0	11	55	N.D.
50 nM	0	26	73	N.D.
150 nM	0	58	85	N.D.
300 nM	9	62	89	0

These data demonstrate that the oligonucleotides of the present invention are able to inhibit the expression of human apolipoprotein(a) in a dose-dependent fashion.

Example 18**Oil red O stain:**

Hepatic steatosis, or clearing of lipids from the liver, is assessed by routine histological analysis of frozen liver tissue sections stained with oil red O stain, which is commonly used to visualize lipid deposits, and counterstained with hematoxylin and eosin, to visualize nuclei and cytoplasm, respectively.

Example 19 Animal models

In addition to human systems, which express apolipoprotein (a), biological systems of other mammals are also available for studies of expression products of the LPA gene as well as for studies of the Lp(a) particles and their role in physiologic processes.

Transgenic mice which express human apolipoprotein(a) have been engineered (Chiesa et al., *J. Biol. Chem.*, **1992**, 267, 24369-24374) and are used as an animal model for the investigation of the *in vivo* activity of the oligonucleotides of this invention. Although transgenic mice expressing human apolipoprotein(a) exist, they fail to assemble Lp(a) particles because of the inability of human apolipoprotein(a) to associate with mouse apolipoprotein B. When mice expressing human apolipoprotein(a) are bred to mice expressing human apolipoprotein B, the Lp(a) particle is efficiently assembled (Callow et al., *Proc. Natl. Acad. Sci. USA*, **1994**, 91, 2130-2134). Accordingly mice expressing both human apolipoprotein(a) and human apolipoprotein B transgenes are used for animal model studies in which the secretion of the Lp(a) particle is evaluated.

Where additional genetic alterations are necessary, mice with either a single human transgene (human apolipoprotein(a) or human apolipoprotein B) or both human transgenes (human apolipoprotein(a) and human apolipoprotein B) are bred to mice with a desired genetic mutation. The offspring with the desired combination of transgene(s) and genetic mutation(s) is selected for use as an animal model. In one nonlimiting example, mice expressing both human apolipoprotein(a) and human apolipoprotein B are bred to mice with a mutation in the leptin gene, yielding offspring producing human Lp(a) particles in an ob/ob model of obesity and diabetes.

ob/ob mice

Leptin is a hormone produced by fat that regulates appetite. Deficiencies in this hormone in both humans and non-human animals leads to obesity. ob/ob mice have a mutation in the leptin gene which results in obesity and hyperglycemia. As such, these mice are a useful model for the investigation of obesity and treatments designed to reduce obesity.

Seven-week old male C57Bl/6J-Lep ob/ob mice (Jackson Laboratory, Bar Harbor, ME) are fed a diet with a fat content of 10-15% and are subcutaneously injected with oligonucleotides of the present invention or a control oligonucleotide at a dose of 5, 10 or 25 mg/kg two times per week for 4 weeks. Saline-injected animals and leptin wildtype littermates (i.e. lean littermates) serve as controls. After the treatment period, mice are sacrificed and target levels are evaluated in liver, brown adipose tissue (BAT) and white adipose tissue (WAT). RNA

isolation and target mRNA expression level quantitation are performed as described by other examples herein.

To assess the physiological effects resulting from antisense inhibition of target apolipoprotein(a) mRNA, the ob/ob mice that receive antisense oligonucleotide treatment are further evaluated at the end of the treatment period for serum lipids, serum apolipoproteins, serum free fatty acids, serum cholesterol (CHOL), liver triglycerides, and fat tissue triglycerides. Serum components are measured on routine clinical diagnostic instruments. Tissue triglycerides are extracted using an acetone extraction technique known in the art, and subsequently measured by ELISA. The presence of the Lp(a) particle in the serum is measured using a commercially available ELISA kit (ALerCHEK Inc., Portland, ME). Hepatic steatosis, or clearing of lipids from the liver, is assessed by measuring the liver triglyceride content. Hepatic steatosis is also assessed by routine histological analysis of frozen liver tissue sections stained with oil red O stain, which is commonly used to visualize lipid deposits, and counterstained with hematoxylin and eosin, to visualize nuclei and cytoplasm, respectively.

The effects of apolipoprotein(a) inhibition on glucose and insulin metabolism are also evaluated in the ob/ob mice treated with antisense oligonucleotides of this invention. Plasma glucose is measured at the start of the antisense oligonucleotide treatment and after 2 weeks and 4 weeks of treatment. Plasma insulin is similarly at the beginning to of the treatment, and following 2 weeks and 4 weeks of treatment. Glucose and insulin tolerance tests are also administered in fed and fasted mice. Mice receive intraperitoneal injections of either glucose or

insulin, and the blood glucose and insulin levels are measured before the insulin or glucose challenge and at 15, 20 or 30 minute intervals for up to 3 hours.

5 To assess the metabolic rate of ob/ob mice treated with antisense oligonucleotides of this invention, the respiratory quotient and oxygen consumption of the mice are also measured.

10 The ob/ob mice that received antisense oligonucleotide treatment are further evaluated at the end of the treatment period for the effects of apolipoprotein(a) inhibition on the expression of genes that participate in lipid metabolism, cholesterol biosynthesis, fatty acid oxidation, fatty acid storage, gluconeogenesis and glucose metabolism. These genes
15 include, but are not limited to, HMG-CoA reductase, acetyl-CoA carboxylase 1 and acetyl-CoA carboxylase 2, carnitine palmitoyltransferase I and glycogen phosphorylase, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 1, lipoprotein lipase
20 and hormone sensitive lipase. mRNA levels in liver and white and brown adipose tissue are quantitated by real-time PCR as described in other examples herein, employing primer-probe sets that were generated using published sequences of each gene of interest.

25

db/db mice

A deficiency in the leptin hormone receptor mice also results in obesity and hyperglycemia. These mice are referred to as db/db mice and, like the ob/ob mice, are
30 used as a mouse model of obesity.

Seven-week old male C57Bl/6J-Lepr db/db mice (Jackson Laboratory, Bar Harbor, ME) are fed a diet with a fat

content of 15-20% and are subcutaneously injected with oligonucleotides of this invention or a control oligonucleotide at a dose of 5, 10 or 25 mg/kg two times per week for 4 weeks. Saline-injected animals and leptin receptor wildtype littermates (i.e. lean littermates) serve as controls. After the treatment period, mice are sacrificed and apolipoprotein(a) levels are evaluated in liver, brown adipose tissue (BAT) and white adipose tissue (WAT). RNA isolation and apolipoprotein(a) mRNA expression level quantitation are performed as described by other examples herein.

After the treatment period, mice are sacrificed and apolipoprotein(a) levels are evaluated in liver, brown adipose tissue (BAT) and white adipose tissue (WAT). RNA isolation and apolipoprotein(a) mRNA expression level quantitation are performed as described by other examples herein.

To assess the physiological effects resulting from antisense inhibition of apolipoprotein(a) mRNA, the db/db mice that receive antisense oligonucleotide treatment are further evaluated at the end of the treatment period for serum lipids, serum apolipoproteins, serum free fatty acids, serum cholesterol (CHOL), liver triglycerides, and fat tissue triglycerides. Serum components are measured on routine clinical diagnostic instruments. Tissue triglycerides are extracted using an acetone extraction technique known in the art, and subsequently measured by ELISA. The presence of the Lp(a) particle in the serum is measured using a commercially available ELISA kit (ALerCHEK Inc., Portland, ME). Hepatic steatosis, or clearing of lipids from the liver, are assessed by measuring the liver triglyceride content. Hepatic

steatosis is also assessed by routine histological analysis of frozen liver tissue sections stained with oil red O stain, which is commonly used to visualize lipid deposits, and counterstained with hematoxylin and eosin, to visualize nuclei and cytoplasm, respectively.

The effects of apolipoprotein(a) inhibition on glucose and insulin metabolism are also evaluated in the db/db mice treated with antisense oligonucleotides. Plasma glucose is measured at the start of the antisense oligonucleotide treatment and after 2 weeks and 4 weeks of treatment. Plasma insulin is similarly at the beginning to of the treatment, and following 2 weeks and 4 weeks of treatment. Glucose and insulin tolerance tests are also administered in fed and fasted mice. Mice receive intraperitoneal injections of either glucose or insulin, and the blood glucose levels are measured before the insulin or glucose challenge and 15, 30, 60, 90 and 120 minutes following the injection.

To assess the metabolic rate of db/db mice treated with antisense oligonucleotides, the respiratory quotient and oxygen consumption of the mice are also measured.

The db/db mice that received antisense oligonucleotide treatment are further evaluated at the end of the treatment period for the effects of apolipoprotein(a) inhibition on the expression of genes that participate in lipid metabolism, cholesterol biosynthesis, fatty acid oxidation, fatty acid storage, gluconeogenesis and glucose metabolism. These genes include, but are not limited to, HMG-CoA reductase, acetyl-CoA carboxylase 1 and acetyl-CoA carboxylase 2, carnitine palmitoyltransferase I and glycogen phosphorylase, glucose-6-phosphatase and

phosphoenolpyruvate carboxykinase 1, lipoprotein lipase and hormone sensitive lipase. mRNA levels in liver and white and brown adipose tissue are quantitated by real-time PCR as described in other examples herein, employing
5 primer-probe sets that were generated using published sequences of each gene of interest.

Lean mice

C57Bl/6 mice are maintained on a standard rodent diet
10 and are used as control (lean) animals. Seven-week old male C57Bl/6 mice are fed a diet with a fat content of 4% and are subcutaneously injected with oligonucleotides of this invention or control oligonucleotide at a dose of 5, 10 or 25 mg/kg two times per week for 4 weeks. Saline-
15 injected animals serve as a control. After the treatment period, mice are sacrificed and apolipoprotein(a) levels are evaluated in liver, brown adipose tissue (BAT) and white adipose tissue (WAT). RNA isolation and apolipoprotein(a) mRNA expression level quantitation are
20 performed as described by other examples herein.

To assess the physiological effects resulting from antisense inhibition of apolipoprotein(a) mRNA, the lean mice that receive antisense oligonucleotide treatment are further evaluated at the end of the treatment period for
25 serum lipids, serum free fatty acids, serum cholesterol (CHOL), liver triglycerides, and fat tissue triglycerides. Serum components are measured on routine clinical diagnostic instruments. Tissue triglycerides are extracted using an acetone extraction technique known in
30 the art, and subsequently measured by ELISA. The presence of the Lp(a) particle in the serum is measured using a commercially available ELISA kit (ALerCHEK Inc., Portland,

ME). Hepatic steatosis, i.e., clearing of lipids from the liver, is assessed by measuring the liver triglyceride content. Hepatic steatosis is also assessed by routine histological analysis of frozen liver tissue sections stained with oil red O stain, which is commonly used to visualize lipid deposits, and counterstained with hematoxylin and eosin, to visualize nuclei and cytoplasm, respectively.

The effects of apolipoprotein(a) inhibition on glucose and insulin metabolism are also evaluated in the lean mice treated with antisense oligonucleotides of this invention. Plasma glucose is measured at the start of the antisense oligonucleotide treatment and after 2 weeks and 4 weeks of treatment. Plasma insulin is similarly at the beginning to of the treatment, and following 2 weeks and 4 weeks of treatment. Glucose and insulin tolerance tests are also administered in fed and fasted mice. Mice receive intraperitoneal injections of either glucose or insulin, and the blood glucose levels are measured before the insulin or glucose challenge and 15, 30, 60, 90 and 120 minutes following the injection.

To assess the metabolic rate of lean mice treated with antisense oligonucleotides of this invention, the respiratory quotient and oxygen consumption of the mice can also be measured.

The lean mice that received antisense oligonucleotide treatment are further evaluated at the end of the treatment period for the effects of apolipoprotein(a) inhibition on the expression of genes that participate in lipid metabolism, cholesterol biosynthesis, fatty acid oxidation, fatty acid storage, gluconeogenesis and glucose metabolism. These genes include, but are not limited to,

HMG-CoA reductase, acetyl-CoA carboxylase 1 and acetyl-CoA carboxylase 2, carnitine palmitoyltransferase I and glycogen phosphorylase, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 1, lipoprotein lipase and hormone sensitive lipase. mRNA levels in liver and white and brown adipose tissue are quantitated by real-time PCR as described in other examples herein, employing primer-probe sets that were generated using published sequences of each gene of interest.

What is claimed is:

1. A compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein(a), wherein said compound is at least 70% complementary to said nucleic acid molecule encoding apolipoprotein(a), and wherein said compound inhibits the expression of apolipoprotein(a) mRNA by at least 10%.
2. The compound of claim 1 comprising 12 to 50 nucleobases in length.
3. The compound of claim 2 comprising 15 to 30 nucleobases in length.
4. The compound of claim 1 comprising an oligonucleotide.
5. The compound of claim 4 comprising an antisense oligonucleotide.
6. The compound of claim 4 comprising a DNA oligonucleotide.
7. The compound of claim 4 comprising an RNA oligonucleotide.
8. The compound of claim 4 comprising a chimeric oligonucleotide.

9. The compound of claim 4 wherein at least a portion of said compound hybridizes with RNA to form an oligonucleotide-RNA duplex.

10. The compound of claim 1 having at least 80% complementarity with said nucleic acid molecule encoding apolipoprotein(a).

11. The compound of claim 1 having at least 90% complementarity with said nucleic acid molecule encoding apolipoprotein(a).

12. The compound of claim 1 having at least 95% complementarity with said nucleic acid molecule encoding apolipoprotein(a).

13. The compound of claim 1 having at least 99% complementarity with said nucleic acid molecule encoding apolipoprotein(a).

14. The compound of claim 1 having at least one modified internucleoside linkage, sugar moiety, or nucleobase.

15. The compound of claim 1 having at least one 2'-O-methoxyethyl sugar moiety.

16. The compound of claim 1 having at least one phosphorothioate internucleoside linkage.

17. The compound of claim 1 having at least one 5-methylcytosine.

18. The compound of claim 1 that preferentially binds to a nucleic acid sequence of apolipoprotein(a) and does not bind, or binds weakly to a nucleic acid sequence encoding plasminogen.

19. The compound of claim 1, wherein said compound comprises a sequence selected from the group consisting of SEQ ID NOs 11, 12, 14, 16, 17, 18, 19, 21, 23, 25, 30, 31, 32, 33, 34, 35, 36, 38, 39, 40, 41, 42, 43 and 45.

20. The compound of claim 1, wherein said compound comprises an antisense nucleic acid molecule that is specifically hybridizable with a 5'-untranslated region (5'UTR) of a nucleic acid molecule encoding apolipoprotein(a).

21. The compound of claim 1, wherein said compound comprises an antisense nucleic acid molecule that is specifically hybridizable with a start region of a nucleic acid molecule encoding apolipoprotein(a).

22. The compound of claim 1, wherein said compound comprises an antisense nucleic acid molecule that is specifically hybridizable with a coding region of a nucleic acid molecule encoding apolipoprotein(a).

23. The compound of claim 1, wherein said compound comprises an antisense nucleic acid molecule that is specifically hybridizable with a stop region of a nucleic acid molecule encoding apolipoprotein(a).

24. The compound of claim 1, wherein said compound comprises an antisense nucleic acid molecule that is specifically hybridizable with a 3'-untranslated region of a nucleic acid molecule encoding apolipoprotein(a).

25. A method of inhibiting the expression of apolipoprotein(a) in a cell or tissue comprising contacting said cell or tissue with the compound of claim 1 so that expression of apolipoprotein(a) is inhibited.

26. A method of screening for a modulator of apolipoprotein(a), the method comprising the steps of:
contacting a preferred target segment of a nucleic acid molecule encoding apolipoprotein(a) with one or more candidate modulators of apolipoprotein(a), and
identifying one or more modulators of apolipoprotein(a) expression which modulate the expression of apolipoprotein(a).

27. The method of claim 26 wherein the modulator of apolipoprotein(a) expression comprises an oligonucleotide, an antisense oligonucleotide, a DNA oligonucleotide, an RNA oligonucleotide, an RNA oligonucleotide having at least a portion of said RNA oligonucleotide capable of hybridizing with RNA to form an oligonucleotide-RNA duplex, or a chimeric oligonucleotide.

28. A diagnostic method for identifying a disease state comprising identifying the presence of apolipoprotein(a) in a sample using at least one of the primers comprising SEQ ID NOs 5 or 6, or the probe comprising SEQ ID NO: 7.

29. A kit or assay device comprising the compound of claim 1.

30. A method of treating an animal having a disease or condition associated with apolipoprotein(a) comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of apolipoprotein(a) is inhibited.

31. The method of claim 24 wherein said compound is a compound of claim 18.

32. The method of claim 24, wherein the disease or condition is a cardiovascular disorder, atherosclerosis, hypercholesterolemia, coronary artery disease or any combination thereof.

33. A method of inhibiting the expression of apolipoprotein(a) comprising contacting a biological system expressing human apolipoprotein(a) with a synthetic antisense compound, wherein said synthetic antisense compound comprises from 15 to 30 nucleobases in length and has at least 3 mismatches to human plasminogen.

34. The method of claim 33 wherein the biological system is a human.

35. The method of claim 33 wherein the biological system is a transgenic animal.

ABSTRACT

Compounds, compositions and methods are provided for
modulating the expression of apolipoprotein(a). The
5 compositions comprise oligonucleotides, targeted to
nucleic acid encoding apolipoprotein(a). Methods of using
these compounds for modulation of apolipoprotein(a)
expression and for diagnosis and treatment of disease
associated with expression of apolipoprotein(a) are
10 provided.

SEQUENCE LISTING

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Mark J. Graham

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gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc	3849
Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser	
1255 1260 1265	
gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc tac cat	3897
Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His	
1270 1275 1280	
ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc aca gga	3945
Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly	
1285 1290 1295 1300	

aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg	3993
Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg	
1305 1310 1315	
acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg	4041
Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg	
1320 1325 1330	
aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt	4089
Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly	
1335 1340 1345	
gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg	4137
Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly	
1350 1355 1360	
act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct	4185
Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala	
1365 1370 1375 1380	
cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc	4233
Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys	
1385 1390 1395	
tac cat ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc	4281
Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val	
1400 1405 1410	
aca gga aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat	4329
Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His	
1415 1420 1425	
agt cgg acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac	4377
Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr	
1430 1435 1440	
tgc agg aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat	4425
Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp	

1445	1450	1455	1460	
ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca				4473
Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala				
1465		1470	1475	
gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta				4521
Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu				
1480	1485		1490	
gag gct cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag				4569
Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln				
1495	1500		1505	
gag tgc tac cat ggt aat gga cag agt tat cga ggc aca tac tcc acc				4617
Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr				
1510	1515		1520	
act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca cca cac				4665
Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His				
1525	1530	1535	1540	
tcg cat agt cgg acc cca gaa tac tac cca aat gct ggc ttg atc atg				4713
Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met				
1545	1550		1555	
aac tac tgc agg aat cca gat gct gtg gca gct cct tat tgt tat acg				4761
Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr				
1560	1565		1570	
agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa tgc tca				4809
Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser				
1575	1580		1585	
gac gca gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg gtt cca				4857
Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro				
1590	1595		1600	
agc cta gag gct cct tcc gaa caa gca ccg act gag caa agg cct ggg				4905

Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly
1605 1610 1615 1620

gtg cag gag tgc tac cat ggt aat gga cag agt tat cga ggc aca tac 4953
Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr
1625 1630 1635

tcc acc act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca 5001
Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr
1640 1645 1650

cca cac tcg cat agt cgg acc cca gaa tac tac cca aat gct ggc ttg 5049
Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu
1655 1660 1665

atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct tat tgt 5097
Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys
1670 1675 1680

tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa 5145
Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln
1685 1690 1695 1700

tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg 5193
Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro
1705 1710 1715

gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag caa agg 5241
Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg
1720 1725 1730

cct ggg gtg cag gag tgc tac cat ggt aat gga cag agt tat cga ggc 5289
Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly
1735 1740 1745

aca tac tcc acc act gtc aca gga aga acc tgc caa gct tgg tca tct 5337
Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser
1750 1755 1760

atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca aat gct	5385
Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala	
1765 1770 1775 1780	
ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct	5433
Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro	
1785 1790 1795	
tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg	5481
Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu	
1800 1805 1810	
acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt	5529
Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val	
1815 1820 1825	
acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag	5577
Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu	
1830 1835 1840	
caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag agt tat	5625
Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr	
1845 1850 1855 1860	
cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa gct tgg	5673
Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp	
1865 1870 1875	
tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca	5721
Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro	
1880 1885 1890	
aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca	5769
Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala	
1895 1900 1905	
gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc	5817
Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys	
1910 1915 1920	

aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg	5865
Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro	
1925 1930 1935 1940	
act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg	5913
Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro	
1945 1950 1955	
act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag	5961
Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln	
1960 1965 1970	
agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa	6009
Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln	
1975 1980 1985	
gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac	6057
Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr	
1990 1995 2000	
tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct	6105
Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala	
2005 2010 2015 2020	
gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag	6153
Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu	
2025 2030 2035	
tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg	6201
Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala	
2040 2045 2050	
cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa	6249
Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln	
2055 2060 2065	
gca ccg act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat	6297
Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn	

2070	2075	2080	
gga cag agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc			6345
Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr			
2085	2090	2095	2100
tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca			6393
Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro			
2105	2110	2115	
gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca			6441
Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro			
2120	2125	2130	
gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg			6489
Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg			
2135	2140	2145	
tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc			6537
Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala			
2150	2155	2160	
gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc			6585
Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser			
2165	2170	2175	2180
gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc tac cat			6633
Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His			
2185	2190	2195	
ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc aca gga			6681
Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly			
2200	2205	2210	
aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg			6729
Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg			
2215	2220	2225	
acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg			6777

Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg	
2230	2235 2240
aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt	6825
Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly	
2245	2250 2255 2260
gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg	6873
Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly	
2265	2270 2275
act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct	6921
Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala	
2280	2285 2290
cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc	6969
Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys	
2295	2300 2305
tac cat ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc	7017
Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val	
2310	2315 2320
aca gga aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat	7065
Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His	
2325	2330 2335 2340
agt cgg acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac	7113
Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr	
2345	2350 2355
tgc agg aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat	7161
Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp	
2360	2365 2370
ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca	7209
Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala	
2375	2380 2385

gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta	7257
Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu	
2390 2395 2400	
gag gct cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag	7305
Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln	
2405 2410 2415 2420	
gag tgc tac cat ggt aat gga cag agt tat cga ggc aca tac tcc acc	7353
Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr	
2425 2430 2435	
act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca cca cac	7401
Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His	
2440 2445 2450	
tcg cat agt cgg acc cca gaa tac tac cca aat gct ggc ttg atc atg	7449
Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met	
2455 2460 2465	
aac tac tgc agg aat cca gat gct gtg gca gct cct tat tgt tat acg	7497
Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr	
2470 2475 2480	
agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa tgc tca	7545
Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser	
2485 2490 2495 2500	
gac gca gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg gtt cca	7593
Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro	
2505 2510 2515	
agc cta gag gct cct tcc gaa caa gca ccg act gag caa agg cct ggg	7641
Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly	
2520 2525 2530	
gtg cag gag tgc tac cat ggt aat gga cag agt tat cga ggc aca tac	7689
Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr	
2535 2540 2545	

tcc acc act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr 2550 2555 2560	7737
cca cac tcg cat agt cgg acc cca gaa tac tac cca aat gct ggc ttg Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu 2565 2570 2575 2580	7785
atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct tat tgt Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys 2585 2590 2595	7833
tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln 2600 2605 2610	7881
tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro 2615 2620 2625	7929
gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag cag agg Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg 2630 2635 2640	7977
cct ggg gtg cag gag tgc tac cac ggt aat gga cag agt tat cga ggc Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly 2645 2650 2655 2660	8025
aca tac tcc acc act gtc act gga aga acc tgc caa gct tgg tca tct Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser 2665 2670 2675	8073
atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca aat gct Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala 2680 2685 2690	8121
ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro	8169

2695	2700	2705	
tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg			8217
Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu			
2710	2715	2720	
acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt			8265
Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val			
2725	2730	2735	2740
acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag			8313
Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu			
	2745	2750	2755
caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag agt tat			8361
Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr			
2760	2765	2770	
cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa gct tgg			8409
Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp			
2775	2780	2785	
tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca			8457
Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro			
2790	2795	2800	
aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca			8505
Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala			
2805	2810	2815	2820
gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc			8553
Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys			
	2825	2830	2835
aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg			8601
Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro			
2840	2845	2850	
act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg			8649

Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro	
2855	2860 2865
act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag	8697
Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln	
2870	2875 2880
agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa	8745
Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln	
2885	2890 2895 2900
gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac	8793
Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr	
	2905 2910 2915
tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct	8841
Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala	
	2920 2925 2930
gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag	8889
Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu	
	2935 2940 2945
tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg	8937
Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala	
	2950 2955 2960
cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa	8985
Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln	
	2965 2970 2975 2980
gca ccg act gag cag agg cct ggg gtg cag gag tgc tac cac ggt aat	9033
Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn	
	2985 2990 2995
gga cag agt tat cga ggc aca tac tcc acc act gtc act gga aga acc	9081
Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr	
	3000 3005 3010

tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca	9129
Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro	
3015 3020 3025	
 gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca	9177
Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro	
3030 3035 3040	
 gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg	9225
Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg	
3045 3050 3055 3060	
 tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc	9273
Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala	
3065 3070 3075	
 gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc	9321
Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser	
3080 3085 3090	
 gaa caa gca ccg act gag cag agg cct ggg gtg cag gag tgc tac cac	9369
Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His	
3095 3100 3105	
 ggg aat gga cag agt tat cga ggc aca tac tcc acc act gtc act gga	9417
Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly	
3110 3115 3120	
 aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg	9465
Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg	
3125 3130 3135 3140	
 acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg	9513
Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg	
3145 3150 3155	
 aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt	9561
Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly	
3160 3165 3170	

gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly 3175 3180 3185	9609
act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala 3190 3195 3200	9657
cct tcc gaa caa gca ccg act gag cag agg cct ggg gtg cag gag tgc Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys 3205 3210 3215 3220	9705
tac cac ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val 3225 3230 3235	9753
act gga aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His 3240 3245 3250	9801
agt cgg acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr 3255 3260 3265	9849
tgc agg aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp 3270 3275 3280	9897
ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala 3285 3290 3295 3300	9945
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Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr			
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Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His			
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Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met			
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aac tac tgc agg aat cca gat cct gtg gca gcc cct tat tgt tat acg			10233
Asn Tyr Cys Arg Asn Pro Asp Pro Val Ala Ala Pro Tyr Cys Tyr Thr			
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Arg Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser			
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Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Ile Thr Pro Ile Pro			
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Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly			
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Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Gln Gly Thr Tyr			
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Phe Ile Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr			
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Pro His Ser His Ser Arg Thr Pro Ala Tyr Tyr Pro Asn Ala Gly Leu	
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Ile Lys Asn Tyr Cys Arg Asn Pro Asp Pro Val Ala Ala Pro Trp Cys	
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Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Thr Arg	
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Cys Ser Asp Ala Glu Trp Thr Ala Phe Val Pro Pro Asn Val Ile Leu	
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Ala Pro Ser Leu Glu Ala Phe Phe Glu Gln Ala Leu Thr Glu Glu Thr	
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Trp Cys Tyr Thr Met Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu	
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Gln Ser Pro Gly Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser Tyr	
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Ser Pro Trp Cys Tyr Thr Met Asp Pro Asn Val Arg Trp Glu Tyr Cys	
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Tyr Thr Met Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Thr Arg			
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Cys Pro Val Thr Glu Ser Ser Val Leu Thr Thr Pro Thr Val Ala Pro			
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Ile Ser Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ser Trp Ser Ser			
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Gly Leu Thr Glu Asn Tyr Cys Arg Asn Pro Asp Ser Gly Lys Gln Pro			
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Trp Cys Tyr Thr Thr Asp Pro Cys Val Arg Trp Glu Tyr Cys Asn Leu			
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Lys Ser Ser Arg Pro Ser Ser Tyr Lys Val Ile Leu Gly Ala His Gln	
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27

DOCKET NO. ISPH-0595US.P1 CUSTOMER NO. 36441
APPLICATION DATA SHEET

PATENT

Application Information	
Application Number::	
Filing Date::	Herewith
Application Type::	Regular
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R::	None
Number of CD disks::	
Number of Copies of CDs::	
Sequence Submission?::	Yes
Computer Readable Form (CRF)?::	Yes
Number of Copies of CRF::	1
Title::	MODULATION OF APOLIPOPROTEIN (A) EXPRESSION
Attorney Docket Number::	ISPH-0595US.P1
Request for Early Publication?	No
Request for Non-Publication?	No
Suggested Drawing Figure::	
Total Drawing Sheets::	0
Small Entity::	No
Latin Name::	
Variety Denomination Name::	
Petition Included::	No
Petition Type	
Licensed US Govt. Agency::	
Contract or Grant Number::	
Secrecy Order in Parent Application::	

Applicant Information	
Applicant Authority Type::	Inventor
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Country of Residence::	United States of America
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State or Province of Mailing Address::	California
Country of Mailing Address::	United States of America
Postal or Zip Code of Mailing Address::	19087

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State or Province of Mailing Address::	California
Country of Mailing Address::	United States of America
Postal or Zip Code of Mailing Address::	92672

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Fax Number::	215-540-5818
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Representative Information		
Representative Customer No. 36441	Registration Number	Name

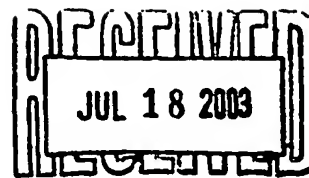
Domestic Priority Information			
Application	Continuity Type	Parent Application	Parent Filing Date
This Application	Is a Continuation-in-part	09/923,515	August 7, 2001
This Application	An application claiming the benefit under 35 USC 119 (e)	60/475,402	June 2, 2003

DOCKET NO. ISPH-0595US.P1 CUSTOMER NO. 36441

PATENT

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Postal or Zip Code of Mailing Address::	92008

PATENT COOPERATION TREATY



From the INTERNATIONAL SEARCHING AUTHORITY

To:
JANE MASSEY LICATA
LICATA & TYRRELL P.C.
66 E. MAIN STREET
MARLTON, NJ 08053

JUL 23 2003

PCT *7/16/03*

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference ISPH-0690	Date of Mailing (day/month/year) 16 JUL 2003
International application No. PCT/US02/24920	International filing date (day/month/year) 05 August 2002 (05.08.2002)
Applicant ISIS PHARMACEUTICALS, INC.	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.
 Filing of amendments and statement under Article 19:
 The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):
 When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.
 Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes
 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 740.14.35
 For more detailed instructions, see the notes on the accompanying sheet.
2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.
3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
 - ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
 - ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.
4. Reminders
 Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 bis.1 and 90 bis.3, respectively, before the completion of the technical preparations for international publication.
 Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.
 In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.
 See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US
Commissioner for Patents
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
Terra C. Gibbs
Telephone No. (703) 308-0196

Janice Ford
for

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference ISPH-0690	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US02/24920	International filing date (<i>day/month/year</i>) 05 August 2002 (05.08.2002)	(Earliest) Priority Date (<i>day/month/year</i>) 07 August 2001 (07.08.2001)
Applicant ISIS PHARMACEUTICALS, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the Report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:



contained in the international application in written form.



filed together with the international application in computer readable form.



furnished subsequently to this Authority in written form.



furnished subsequently to this Authority in computer readable form.



the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.



the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☒ Unity of invention is lacking (See Box II).

4. With regard to the title,



the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the abstract,



the text is approved as submitted by the applicant.



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No. _____



as suggested by the applicant.



because the applicant failed to suggest a figure.



because this figure better characterizes the invention.



None of the figures

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/24920

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: Claims 1-20, SEQ ID NOs 7, 8, 19 and 36
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/24920

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; A01N 43/04; C07H 21/04, A61K 31/07
US CL : 435/6, 325, 91.1, 375; 536/24.5, 23.1, 24.3, 24.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 325, 91.1, 375; 536/24.5, 23.1, 24.3, 24.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Biosis, Medline, CaPlus, Embase, Cancerlit

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOSTNER et al. Lipoprotein (a): Still an enigma? Current Opinion in Lipidology. 2002, Vol. 13, pages 391-396.	15-20
A	JEN ET AL. Suppression of Gene Expression by Targeted Disruption of Messenger RNA: Available Options and Current Strategies. Stem Cells, 2000 Vol. 18, pages 307-317.	15-20
A	BRANCH, A. A Good Antisense Molecule is Hard to Find. TIBS. February 1998, Vol. 23, pages 45-50.	15-20
Y	McLEAN et al. cDNA sequence of human apolipoprotein (a) is homologous to plasminogen. Nature. 1987, Vol. 330, pages 132-137.	1-15
Y	WEINTRAUB, H.M. Antisense RNA and DNA. Scientific American. January 1990, pages 40-46, see entire article.	1-15
Y	MILLIGAN et al. Current Concepts in Antisense Drug Design. Medicinal Chemistry. 1993, Vol. 36, pages 1923-1937.	1-15
Y	US 5,801,154 A (BARACCHINI et al) 01 September 1998, see column 7 lines 6 and 22 and column 8 line 12; column 6 lines 12-17 and (column 4 lines 26-30.	1-15
Y	FRITZ et al. Cationic Polystyrene Nanoparticles: Preparation and Characterization of a Model Drug Carrier System for Antisense Oligonucleotides. Journal of Colloid and Interface Science. 1997, Vol. 195, pages 272-288.	1-15

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

<p>Special categories of cited documents:</p> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier application or patent published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p>	<p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>*Z* document member of the same patent family</p>
---	---

Date of the actual completion of the international search

Date of mailing of the international search report

03 February 2003 (03.02.2003)

16 JUL 2003

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

Terra C. Gibbs

Telephone No. (703) 308-0196

Facsimile No. (703)305-3230

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US02/24920

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ✓	FRANK et al. Adenovirus-mediated apo(a) antisense RNA expression efficiently inhibits apo(a) synthesis in vitro and in vivo. Gene Therapy. 2001, Vol. 8, pages 425-430.	1-15
X ✗	MORISHITA et al. Novel Therapeutic Strategy for Atherosclerosis: Ribozyme Oligonucleotides against apo(a) selectively inhibit apo(a) but not plasminogen gene expression. Circulation. 1998, vol. 98, pages 1898-1904.	1-15
X ✓	WO 96/009392 A1 (RIBOZYME PHARMACEUTICALS, INC.) 28 March 1996.	1-15

INTERNATIONAL SEARCH REPORT

PCT/US02/24920

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-20, SEQ ID NO: 7

Group II, claims 1-20, SEQ ID NO: 8

Group III, claims 1-20, SEQ ID NO: 9

Group IV, claims 1-20, SEQ ID NO: 10

Group V, claims 1-20, SEQ ID NO: 11

Group VI, claims 1-20, SEQ ID NO: 12

Group VII, claims 1-20, SEQ ID NO: 13

Group VIII, claims 1-20, SEQ ID NO: 14

Group IX, claims 1-20, SEQ ID NO: 15

Group X, claims 1-20, SEQ ID NO: 16

Group XI, claims 1-20, SEQ ID NO: 17

Group XII, claims 1-20, SEQ ID NO: 18

Group XIII, claims 1-20, SEQ ID NO: 19

Group XIV, claims 1-20, SEQ ID NO: 20

Group XV, claims 1-20, SEQ ID NO: 21

Group XVI, claims 1-20, SEQ ID NO: 22

Group XVII, claims 1-20, SEQ ID NO: 23

Group XVIII, claims 1-20, SEQ ID NO: 24

Group XIX, claims 1-20, SEQ ID NO: 25

Group XX, claims 1-20, SEQ ID NO: 26

Group XXI, claims 1-20, SEQ ID NO: 27

Group XXII, claims 1-20, SEQ ID NO: 28

Group XXIII, claims 1-20, SEQ ID NO: 29

Group XXIV, claims 1-20, SEQ ID NO: 30

INTERNATIONAL SEARCH REPORT

PCT/US02/24920

Group XXV, claims 1-20, SEQ ID NO: 31

Group XXVI, claims 1-20, SEQ ID NO: 32

Group XXVII, claims 1-20, SEQ ID NO: 33

Group XXVIII, claims 1-20, SEQ ID NO: 34

Group XXIX, claims 1-20, SEQ ID NO: 35

Group XXX, claims 1-20, SEQ ID NO: 36

Group XXXI, claims 1-20, SEQ ID NO: 37

Group XXXII, claims 1-20, SEQ ID NO: 38

Group XXXIII, claims 1-20, SEQ ID NO: 39

Group XXXIV, claims 1-20, SEQ ID NO: 40

Group XXXV, claims 1-20, SEQ ID NO: 41

As outlined above, this international searching authority has found 35 inventions claimed in the International Application covered by the claims indicated: Claims 1-20 which specifically claim sequences listed as SEQ ID NOs 7-41, which are intended to modulate the function and/or expression of human apolipoprotein a.

This international searching authority considers that the international application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups 1-XXXV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

According to the guidelines in Section (f)(i)(a) of Annex B of the PCT Administrative Instructions, the special technical feature as defined by PCT Rule 13.2 shall be considered to be met when all the alternatives of a Markush-group are of similar nature. For chemical alternatives, such as the claimed antisense sequences, the Markush group shall be regarded as being of similar nature when

- (A) all alternatives have a common property or activity and
- (B)(1) a common structure is present, i.e., a significant structure is shared by all of the alternatives or
- (B)(2) in cases where the common structure cannot be the unifying criteria, all alternatives belong to an art recognized class of compounds in the art to which the invention pertains.

The instant antisense sequences are considered to be each separate inventions for the following reasons:

The sequences do not meet the criteria of (A), common property or activity or (B)(2), art recognized class of compounds. Although the sequence target and modulate expression of the same gene, each antisense sequence behaves in a different way in the context of the claimed invention. Each sequence targets a different and specific region of gene Y and each sequence modifies (either increases or decreases) the expression of the gene to varying degrees (per Applicants' Table I in the specification). Each member of the class cannot be substituted, one for the other, with the expectation that the same intended result would be achieved.

Further, although the sequence target the same gene, the sequences do not meet the criteria of (B)(1), as they do not share, one with another, a common core structure. Accordingly, unity of invention between the antisense sequences is lacking and each antisense sequence claimed is considered to constitute a special technical feature.

Applicants will obtain a search of the first sequence listed in the first invention. For every other sequence applicants wish to have searched, applicants need to elect the sequence and pay an additional fee.

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/014540

International filing date: 02 June 2004 (02.06.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 10/684,440
Filing date: 15 October 2003 (15.10.2003)

Date of receipt at the International Bureau: 29 November 2004 (29.11.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

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